PHYTOCHEMICAL INVESTIGATION OF *COLOPHOSPERMUM MOPANE*

By

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PHYTOCHEMICAL INVESTIGATION OF *COLOPHOSPERMUM MOPANE*

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ABSTRACT

A previously partially-characterized diterpene alcohol was isolated from *Colophospermum mopane*, and its structure completely elucidated. A second novel diterpene alcohol, structurally related to the first, was also fully characterized by NMR spectroscopy. A proposed precursor of the diterpenes, a mixed pair of diterpene aldehydes, was isolated, and upon reduction yielded a mixture of both diterpene alcohols. These diterpenes represent important "missing" links in the biogenesis of 9,13-epoxylabdanes.

Seeds of *C. mopane* were grown in greenhouse conditions to determine when these diterpenes were produced. Two sesquiterpenes and two diterpenes were quantified by GC-MS. Seedlings grown from seeds rinsed in hexane grew faster and produced terpenoids sooner than the control group. It is likely that *C. mopane* seeds have terpenoids present in concentrations high enough to minimize competition from herbaceous perennials, at the cost of some degree of auto-toxicity, so rinsing promotes growth and terpenoid production.

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I owe a great deal to Dr. Clausen, easily the best and most patient instructor I have had the privilege of studying under. I am eternally in his debt for his support, his faith in me, and his friendship. (Although I still have mixed feelings about a ferret as a namesake.)

Last but not least, I must thank Timothy Howe. You're my world, love.

1.0. INTRODUCTION

Colophospermum mopane is medium-sized leguminous tree, 5-12 meters tall, that sometimes occurs as a 1-2 meter shrub. It is irregularly deciduous, corresponding to the dry season in May, and the dead or dried leaves may remain on the tree [Timberlake, 1995]. It is found in almost pure strands in hot, low-lying areas of sub-tropical Southern Africa [van Wyk and van Wyk, 1997].

The leaves are alternate and basally articulated, with a single pair of distinctive triangular leaflets, resembling two butterfly wings. There is no midrib, but there are numerous prominent veins arising from the point of attachment. Mature leaves have numerous gland dots on the lamina, or upper leaf surface. The seeds are contained in large flat papery pods, 3-6 cm long. The light-brown pods are reniform or obliquely semi-circular, with scattered resin glands on the surface. The seeds themselves are large, 1-3 centimeters long, have a corrugated appearance, and can weigh up to 0.5 g. They are covered with numerous small, sticky glands, containing a variety of terpenes. The seeds are exceptionally hardy, and may lay dormant for many years before germinating [Smit and Rethman, 1998].

Mopane, as it is commonly called, has a fairly wide distribution in Africa, found from coast to coast between the Tropic of Cancer and 10 degrees South latitude. It is often found on alluvial or lime-rich soils, although it tolerates xeric and low nitrogen or potassium conditions, and is sometimes said to be an indicator of sodic or inferior soils. The seedlings are quite stress tolerant, although they are susceptible to competition, especially from grasses, and are very frost sensitive. There are few typically associated species that occur in mopane woodlands, and little grass or herbaceous plant cover. Mature mopane develops a superficial root system which is able to suppress perennial grasses, and it is not uncommon to observe isolated trees with only sparse annual grasses and herbs under the trees [Bingham, 1995]. The vegetation in these areas is almost exclusively mopane.

Colophospermum mopane is ecologically important for both elephants and humans [Timberlake, 1995]. For elephants, *C. mopane* represents an important browse species, and the elephants play a major role in shaping mopane stands. If the mopane plants are heavily browsed, they will remain in a juvenile shrub growth form. If they are not browsed heavily, the growth form will predominantly be taller trees, also known as "cathedral mopane" [Timberlake, 1995]. Elephants appear to prevent recruitment into the taller size classes, which prevents mopane from growing up out of the reach of most browsing mammals, but they are also able to fell mature trees in order to feed on crown foliage. Elephants have also been observed to preferentially harvest and consume specific plant parts, the leaves, shoots, and bark, from particular individuals and stands of mopane, while leaving others untouched [Dudley, 1999]. Some plant parts, the roots and fruit of mopane, do not seem palatable to elephants, although other principle forage species do not have this distinction. Over the course of a five-year study in Zimbabwe, only one instance each of elephants feeding on roots and seeds was recorded [Dudley, 1999].

The most important human use of *C. mopane* is as the host plant for the edible larvae of the Saturnid moth *Gonimbrasia belina*, known locally as the mopane worm [Timberlake, 1995]. In some impoverished regions, this larvae represents a major percentage of daily protein sources. Harvesting mopane worms is an important source of revenue for the rural poor; the market in South Africa alone runs into thousands of tons [Bartlett, 1997]. Local anthropogenic uses include firewood, charcoal, and timber sources. The wood is too hard for furniture, though it is sometimes used for craftwork and for fence construction. Mopane is also a graze-species for livestock, particularly during the dry season. The leaves alone are not enough for survival of cattle [Timberlake, 1995], and there is anecdotal evidence that a diet consisting solely of mopane leaves results in the death of cattle after only a few days [pers. comm., Joe Dudley].

Reported local uses for *C. mopane* also include medicinal agents [Watt and Breyer-Brandwijk, 1962]. Bark and root extracts are sometimes used to treat aliments ranging from eye inflammation to syphilis to temporary madness. However, no chemical study investigating these claims has ever been published.

Several studies of the secondary metabolites of *Colophospermum mopane* have been undertaken, concentrating mainly on components of the heartwood. A novel class of condensed tannins, phlobatannins, have been found in the heartwood of *C. mopane* [Steenkamp *et al*, 1985], as have di- and triflavanoids [Botha *et al*, 1982; Steynberg *et al*, 1990; Steenkamp *et al*, 1988], and anthocyanidins [Drewes and Roux, 1967]. One additional GC/MS study described the composition of the steam distillate of the bark, leaves, and seeds [Brophy *et al*, 1992]. Most recently, several diterpenes were isolated and characterized from the bark and seeds [Mebe, 2001].

A novel diterpene alcohol, Compound I, was previously isolated and partially characterized by Erica Cederstrom and Edward Treadwell [Treadwell, 1996]. Initial NMR and GC-MS results indicated a saturated 9,13-epoxylabdane with the formula $C_{20}H_{36}O_3$ (Figure 1.1). Two alcohol moieties, one primary and one secondary, were verified by an oxidation experiment, while the final oxygen atom belonged to an ether, in agreement with NMR spectral data [Treadwell, 1996].

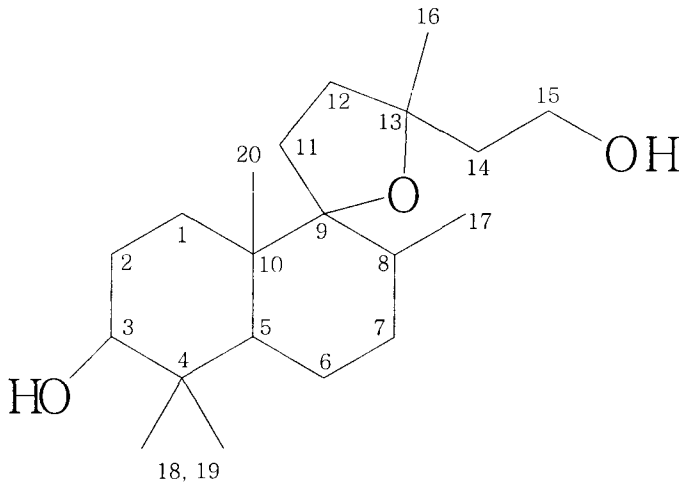

Figure 1.1. Skeleton structure of Compound I.

Complete structural elucidation required the assignment of five stereocenters relative to C-10, which was assumed to be the normal *S* configuration. At the outset of this investigation, only one stereocenter had been unambiguously assigned. Cederstrom and Treadwell assigned the alcohol at C-3 to an alpha position, based on coupling constants in the ^{1}H NMR. If the proton is equatorial, it will have equivalent dihedral angles of 60 degrees to both of the protons on C-2, and the corresponding ^{1}H NMR peak should be a triplet, as is the case with this molecule. The coupling constant is about 3 Hz, which is consistent with an equatorial proton. If the C-3 proton was axial, however, the expected NMR peak would be a doublet of doublets, with expected coupling constant values of approximately 2-3 Hz for the axial-equatorial interaction, and 8-10 Hz for the axial-axial interaction [Silverstein *et al*, 1991].

The bridgehead proton at C-5 and the bridgehead methyl at C-10 were tentatively assigned by Bredlie and Treadwell to axial positions due to several factors. Other naturally-occurring 9,13-epoxylabdanes have a trans-decalin stereochemistry, and the chemical shifts of the NMRs of this compound are consistent with those in the literature [Treadwell, 1996]. Furthermore, this is the stereochemistry favored by the biosynthetic pathway (Figure 1.2).

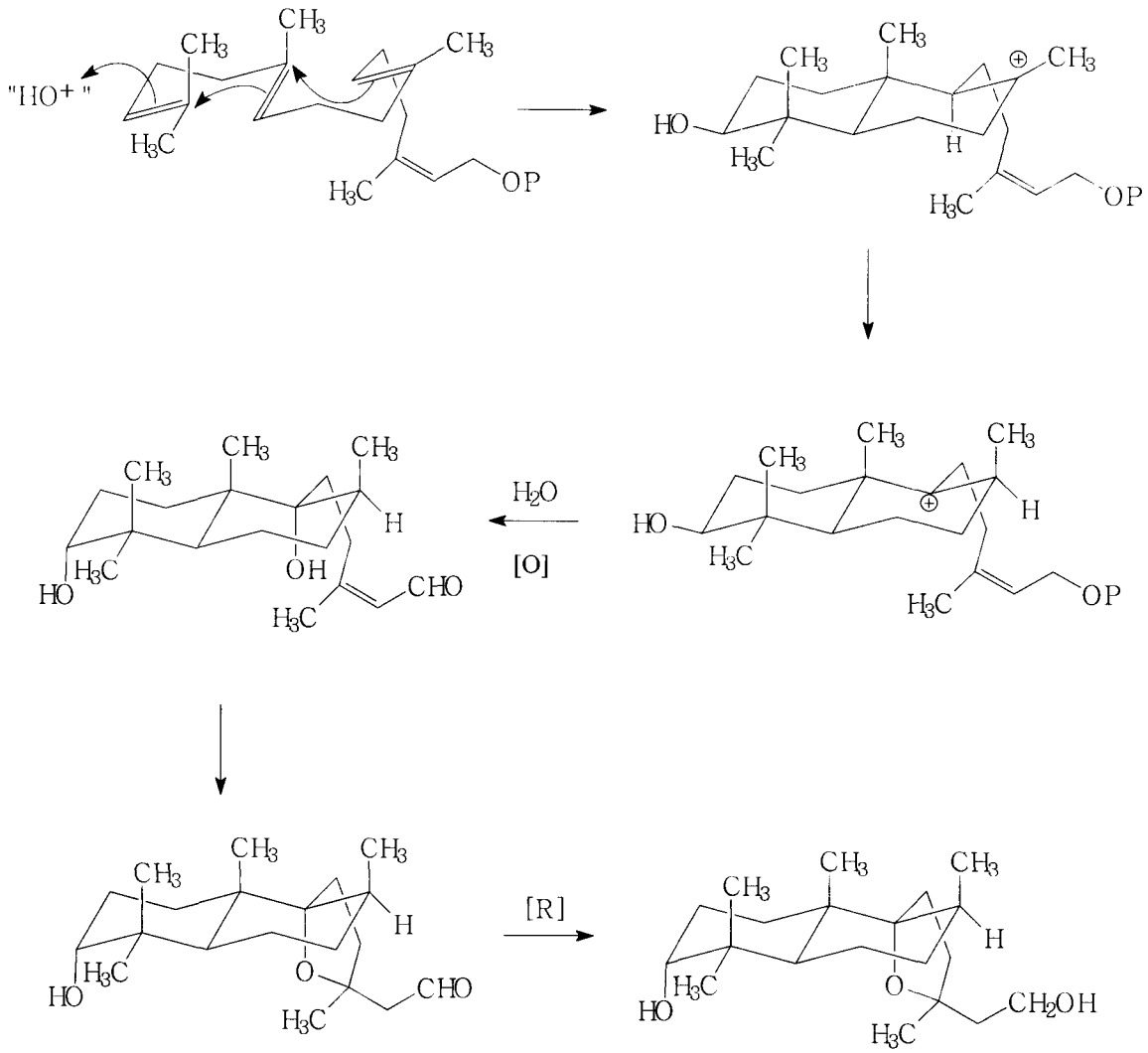

Figure 1.2. Proposed biosynthetic scheme for Compound I from geranylgeranyl phosphate.

Biosynthetic considerations also suggest that the methyl at C-8 is axial. In this instance, there is no clear consensus in the literature. Many labdanes, in particular 9,13-epoxylabdanes, have this methyl in the alpha position [Adinolfi *et al*, 1988; Fulke *et al*, 1968; Hoffman *et al*, 1987]. More recently, however, labdanes with the C-8 methyl in the beta position have been reported, and in most instances the stereochemistry is verified by both NMR spectroscopy and X-ray crystallography [Hashimoto *et al*, 1995; Konishi *et*

al, 1998; Akhila *et al*, 1990; Toyota *et al*, 1988; Paternostro *et al*, 2000; Mebe, 2001] (Figure 1.3).

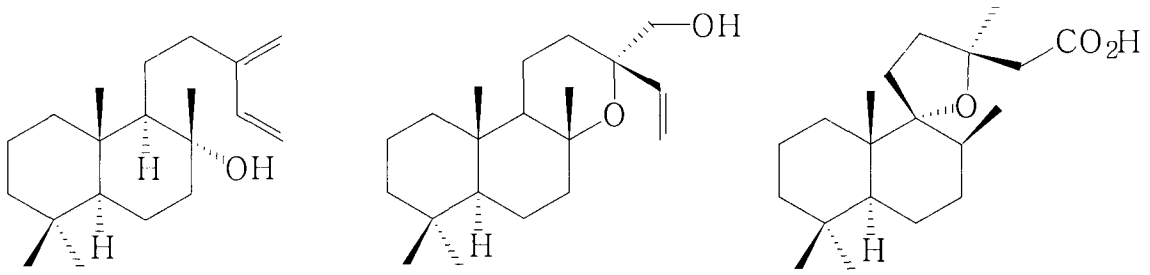

Figure 1.3. Labdane diterpenes with β methyl at C-8 [Paternostro *et al*, 2000; Mebe, 2001].

The configuration at C-9 was tentatively assigned by examining naturally-occurring 9,13-epoxylabdanes [Treadwell, 1996]. All of the compounds published assign the ether linkage alpha. This is consistent with the steric considerations in the proposed mechanism (Figure 1.2).

Therefore, four of the six stereocenters, C-3, C-5, C-9, and C-10, were tentatively assigned prior to this study: centers C-5, C-9 and C-10 were assigned based upon the literature and biosynthetic considerations, and C-3 was clearly shown to be alpha to C-10. The stereocenters C-8 and C-13 had not been assigned. Previous work focused on achieving a crystalline compound suitable for X-ray crystallography to obtain unambiguous structural information. Several derivatives were synthesized in this attempt, including a p-bromobenzoate derivative and a p-iodobenzoate derivative [Treadwell, 1996]. Neither synthesis, however, produced suitable crystals.

The goal of this study was to isolate more of the same compound and further examine it by NMR spectroscopy, as well as to isolate and characterize any related compounds. In particular, a pair of diterpene aldehydes partially characterized by Treadwell [Treadwell,

1996] is of interest. These mixed aldehydes appear to be structurally very similar to Compound I (Figure 1.4), and may provide insight into the biosysnthesis of Compound I.

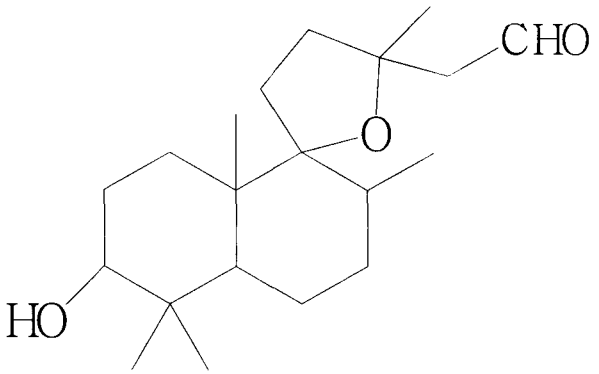

Figure 1.4. Skeleton structure of mixed pair of diterpene aldehydes.

One important avenue in structural determination to pursue is alternative NMR solvents. The main setback to assigning stereochemistry in this molecule has been ambiguities due to overlapping peaks in the ^{1}H NMR. Heterocyclic compounds in general, and cyclic ethers in particular, may experience shifts of up to +0.5 ppm in ^{1}H NMR in d_6-benzene when compared to $CDCl_3$ [Narayanan and Bhadane, 1968]. A change of solvent may allow previously overlapping peaks to be unambiguously observed. In addition, derivatives may be synthesized in order to alter the chemical shifts of some key protons. The structure of Compound I should be solvable by newer, more sensitive NMR experiments, including NOESY, gHMBC, and gHSQC. Furthermore, with the acquisition of a 300-MHz NMR by the Department of Chemistry and Biochemistry at UAF, instrument time and cost of experiments are no longer limiting factors.

A secondary goal of this project involved a study of mopane seedlings in greenhouse conditions. Mopane seeds are heavily coated in resinous glands, which exude a variety of secondary metabolic products. It is possible that the mopane seedlings, after germination, are able to utilize these terpenes as either primary metabolites or as sources of energy. Since terpenes are bioenergetically among the most expensive compounds to synthesize, terpenes are often recycled into primary metabolism [Gershenzon, 1994;

Langenheim, 1994]. Removing this resin, either by manually removing the seed coat or chemically extracting it with an organic solvent, will determine whether the seedlings can reabsorb these terpenes and use them to accelerate growth. Two groups of seedlings will be examined: a control group with the resin ducts intact, and a "rinsed" group without the resinous coating. Samples will be taken at various time intervals, and terpene content analyzed spectroscopically. In particular, the seedlings will be examined for production of Compound I and any precursors from the proposed biosynthetic pathway.

2.0. RESULTS AND DISCUSSION

2.1. Isolation of Diterpenes

2.1.1. Diterpene Alcohol (Compound I). Using the protocol of Cederstrom [Treadwell, 1996], 50 mg of pure Compound I was isolated from the seed husk extract. Initial ^{1}H and ^{13}C NMR, including DEPT, were obtained in $CDCl_3$ to verify that the structure was identical to the previously isolated diterpene alcohol. A mass spectrum with a molecular ion peak of 324.26403 amu was also recorded for further confirmation.

NMR spectra were then acquired in d_6-benzene. Two-dimensional spectra, including gDQCOSY, TOSCY, gHSQC, and gHMBC, were recorded to completely assign all peaks (Figures 2.1-2.3; Table 2.1). There were significant solvent-induced shifts in some of the key regions of the ^{1}H NMR (Table 1). Most importantly, previously overlapping methyls had become more distinct. The C-10 methyl (C-20), was now distinct from C-18 at 0.80 ppm. The C-8 methyl doublet (C-17), shifted from 1.05 ppm to 0.90 ppm, now distinct from the H-1 axial, which shifted from 1.05 ppm to 0.82 ppm. This last shift of the C-17 methyl doublet is particularly important to determining the stereochemistry at this site. Two previously distinct methyls unfortunately now overlapped: the C-13 methyl (C-16), which had been at 1.26 ppm, and the equatorial C-4 methyl (C-18), which was at 1.00 ppm, now overlapped at about 1.03 ppm. However, these methyls were not critical to determining overall stereochemistry.

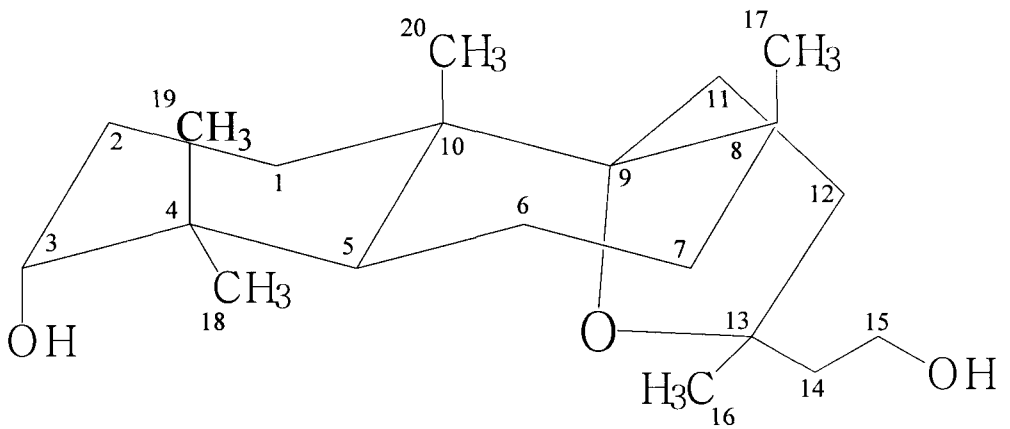

Figure 2.1. Stereochemistry of Compound I.

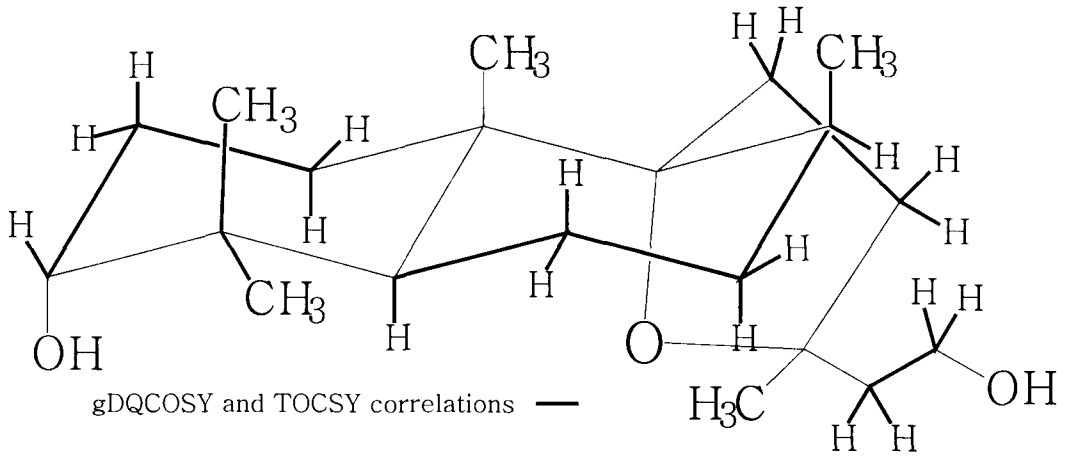

Figure 2.2. COSY and TOCSY connectivities for Compound I.

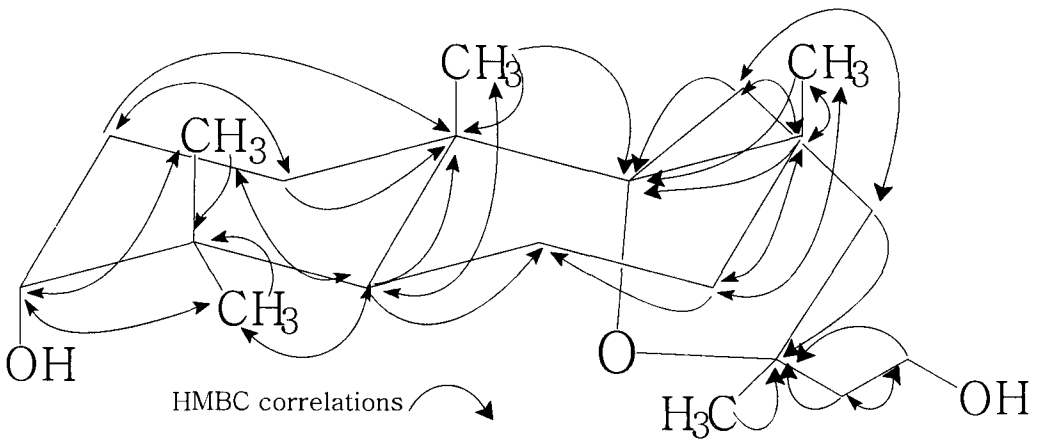

Figure 2.3. HMBC correlations for Compound I.

Other important shifts occurred as well. The C-5 proton shifted downfield to 1.95 ppm, which is more distinct than its previous location, buried under other peaks at 1.65 ppm. The equatorial protons on C-1 and C-7 still overlapped, but the C-14 proton shifted from 2.08 ppm to 1.91 ppm and is no longer is buried in this region. Two diastereotopic groups of protons now appear to be nearly magnetically equivalent: the protons on C-6 now both appear at 1.32 ppm, and the protons on C-12 both shifted to 1.43 ppm.

The 2-dimensional spectra allowed for assignments of most of the protons and carbons in Compound I; however, little additional stereochemical information was obtained. The

next step was to perform NMR experiments for spacial interactions: nuclear Overhauser effect (NOE) difference spectra measurements and 2-dimensional NOESY spectra (Figure 2.4). These spectra also resulted in complete assignments of the remaining protons.

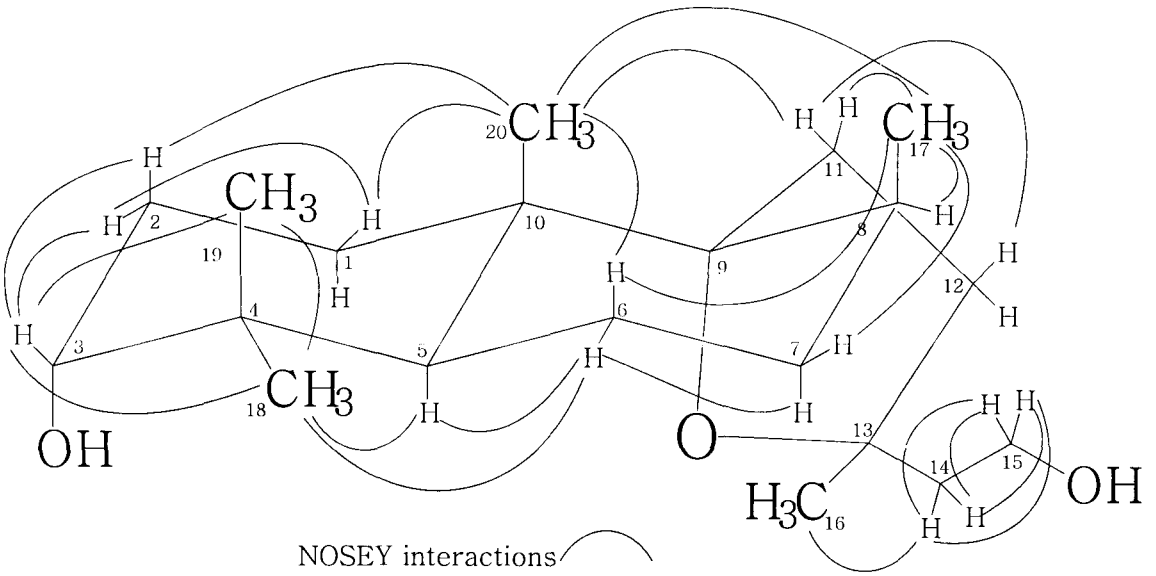

Figure 2.4. NOESY correlations for Compound I.

Irradiation of the proton on C-3 produced an enhancement of the signals of C-18 and C-19 equally. This is consistent with an earlier assignment of an alpha alcohol at C-3 based on ^{1}H NMR coupling constants. In order to produce a triplet in the ^{1}H NMR, the C-3 proton must be in a staggered position with both the C-2 protons and the methyls on C-4. It must therefore be equatorial, setting the alcohol at C-3 axial. It is possible for the C-3 proton to be staggered to both C-2 and C-4 if the ring is in a boat conformation and the alcohol is equatorial. However, in this position there is a great deal of steric hindrance between the C-3 alcohol and C-20. The ring would contort to a twist boat, and the C-3 proton would no longer be staggered to both C-18 and C-19. Hyperchem modeling by both semi-emperical methods (AM1 and PM3) and molecular modeling (MM+) shows that the most energetically favored position is an axial C-3 alcohol and an overall chair-chair configuration (Appendix A).

The only NOE that the proton at C-8 exhibits is with the C-17 methyl doublet. If this proton was axial, a strong NOE with the C-20 methyl would be expected. This is not the case, and so it is very likely that the C-8 proton is equatorial. The chemical shift of the C-8 proton is consistent with that of an equatorial proton, since an axial C-8 proton would be expected to appear more upfield than its current location at 1.75 ppm. There is an NOE between C-17 and the equatorial C-7 proton, but not with the axial C-7 proton. There is also an NOE between C-17 and the C-6 proton, which would not be possible if the C-17 methyl was equatorial. Most importantly, the NOESY spectrum shows a strong correlation between the C-17 methyl and the C-20 methyl. This correlation is only possible if C-17 is axial. Therefore, C-17 must be axial. Hyperchem modeling again confirms that this is the most energetically favored configuration, as opposed to boat and twist-boat forms (Appendix A).

Initial attempts to determine the stereochemistry at C-5 focused on NOE measurements associated with the C-4 gem dimethyls. Therefore, the methyls at C-4 must be assigned. Axial protons usually appear upfield of equatorial protons [Silverstein, 1991], although this can be affected by electrom-withdrawing groups nearby. Therefore C-19, the axial methyl, appears at 0.75 ppm, while the equatorial methyl, C-18, is 1.03 ppm. If a trans-decalin configuration is present, there should be an NOE between C-19 and C-20, and an NOE between C-18 and the proton on C-5. Unfortunately, an NOE between C-19 and C-20, the 1,3-diaxial methyls, is not visible, because the two peaks are too close together in the ^{1}H NMR. Irradiating one peak has the effect of irradiating the other. This problem is not mitigated with the use of high resolution 2-D NOESY; any possible correlation is hidden by the diagonal baseline. There is a strong NOE between C-18 and the C-5 proton, as well as between C-18 and the C-6 protons. No NOE exists between C-19 and the C-5 proton. The NOE between the equatorial C-4 methyl and the C-5 proton, and lack of NOE between the axial C-4 methyl and C-5, serve as tenuous evidence that Compound I is trans-decalin. Further confirmation that the ring system is trans-decalin must be found in other NOE correlations. There is no NOE apparent between the C-20

methyl and the C-5 proton; therefore, these two cannot be on the same side of the ring. Final proof of a trans-decalin system is seen in the NOEs between the C-20 methyl and the C-17 methyl and between C-20 and one of the C-6 protons.

NOEs also showed that the ether linkage is alpha to the decalin ring system, following the precedent set in the literature [Treadwell, 1996]. An NOE is observed with a C-11 proton (1.68 ppm) when C-20 is irradiated, and the opposite C-11 proton (1.50 ppm) shows an NOE with C-17. If the ether linkage was beta to the ring, no NOEs would be observed between C-11 and the C-17 and C-20 methyl groups. These last two NOEs also allow for unambiguous assignment of the diastereotopic C-11 protons.

The last stereocenter to be assigned, C-13, presented a challenge. The C-13 carbon with the C-16 methyl and 2-hydroxyethyl group are not proximal to the decalin system, and so NOE experiments focusing on C-1, C-2, C-7, and C-8 did not solve the problem. The protons on C-12 are magnetically equivalent (1.43 ppm), so NOE correlations cannot be followed to determine stereochemistry through C-11 and C-12 to the decalin ring system.

Previous work had focused on synthesizing a derivative to obtain a crystal suitable for X-ray crystallography. While these attempts were unsuccessful, examination of the NMR data of the p-bromobenzoate derivative, Compound Ia, (Figure 2.5) revealed insight into the stereochemistry at C-13 (Table 2.2). The chemical shifts of the protons near the benzoate derivative are expected to move downfield due to the magnetic anistropic effects of the aromatic ring, although an upfield shift is sometimes seen. The C-15 protons, the C-14 protons, and the C-16 methyl all experienced a downfield shift. The chemical shift of any proton adjacent to the p-bromobenzoate group is expected to change as well. Two protons in particular moved significantly: the C-8 proton and the C-5 proton. The C-8 proton can shift only if the p-bromobenzoate moiety is adjacent to ring B. Therefore, this stereocenter may be assigned as an *S* configuration: the C-16 methyl is adjacent to ring A, and the 2-hydroxyethyl group is adjacent to ring B.

Interestingly, the effect of benzoylation on the chemical shift of the C-5 proton independently confirms the earlier assignment of a trans-decalin system.

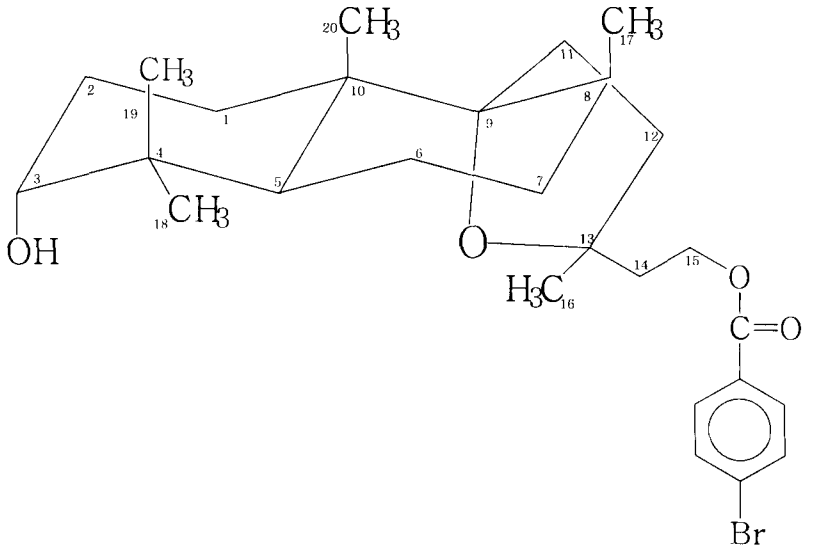

Figure 2.5. Compound Ia, p-bromobenzoate derivative of Compound I.

2.1.2. Alcohol Epimer (Compound II). Seventeen milligrams of a substance with chromatographic behavior similar to the diterpene alcohol (Rf of 0.42 on silica gel eluted with diethyl ether, compared to Compound I at 0.26) was isolated from the seed husk extract. Initial proton NMR revealed a spectrum very similar to Compound I. Five methyl peaks were present, four of which were singlets and one which was a doublet. The ^{13}C NMR and DEPT spectra were also very similar, and showed 20 carbons, with the most downfield at 93.8 ppm. Analysis by GC-MS revealed a molecular ion peak of 324.26565, and a fragmentation pattern almost identical to that of the previously identified diterpene alcohol (Figures 2.6 and 2.7). Distinct differences in the ^{1}H and ^{13}C NMRs, however, confirm that this is a unique diterpene, most likely an epimer of the diterpene alcohol (Table 2.3). In particular, the protons on C-12, which were both located at 1.43 ppm, are now magnetically distinguishable at 1.36 and 1.51 ppm. The C-14 carbon has shifted downfield by 2 ppm, from 42.87 ppm to 44.62 ppm, and the downfield C-14 proton has moved upfield, from 1.91 to 1.74 ppm. Two of the methyls have moved as well: the C-13 methyl carbon shifted from 28.90 to 25.71 ppm and its

protons shifted from 1.04 to 1.13 ppm, and equatorial methyl protons on C-4 have shifted upfield from 1.03 to 0.89 ppm.

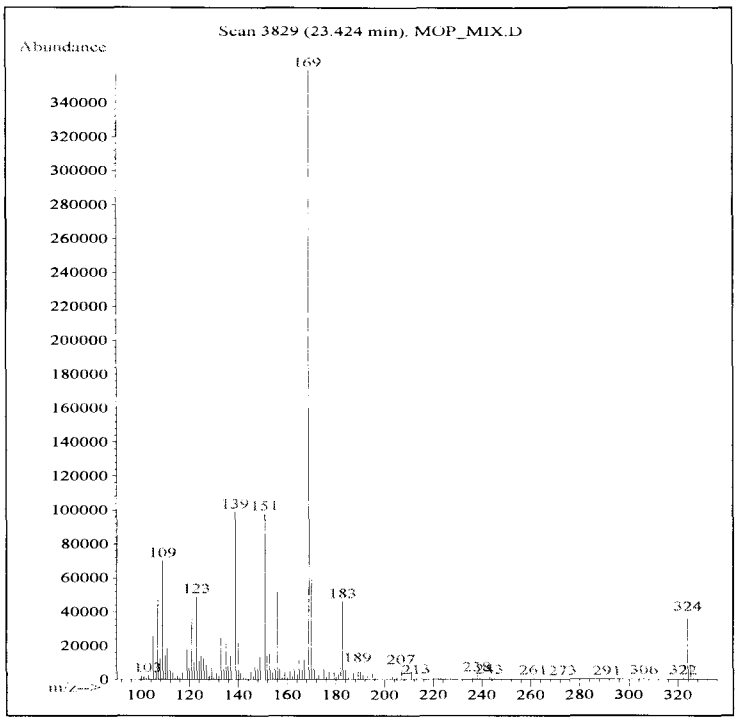

Figure 2.6. EI-MS of Compound I.

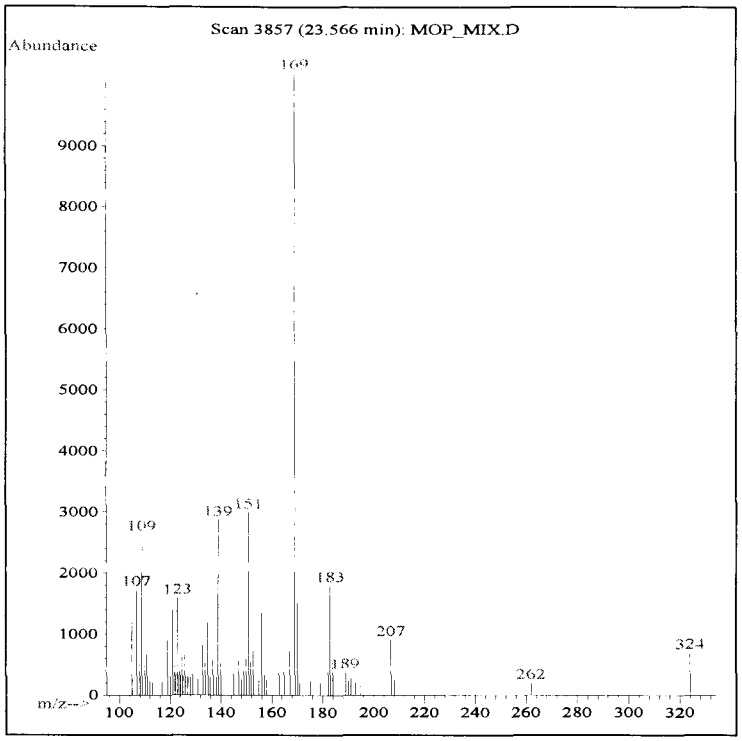

Figure 2.7. EI-MS of Compound II.

Complete assignments were made using COSY, HSQC, and HMBC data (Figures 2.8-2.10). Based on the chemical shift data, the most likely center for epimerization was C-13. This stereocenter has previously been reported in both the *R* and *S* configurations for other 9,13-epoxylabdanes [Rivett, 1976; Jakupovic *et al*, 1986; Adinolfi *et al*, 1988; Zdero *et al*, 1991; Konishi *et al*, 1998]. However, direct evidence for an *R* configuration was not provided by COSY, HSQC, or HMBC experiments. NOE and NOESY spectra were then run on Compound II (Figure 2.11.), but like the difficulties associated with assigning this center in Compound I, NOESY experiments did not provide a clear answer. Since a derivative of Compound I allowed for elucidation of the stereocenter at C-13, it was presumed that a derivative of Compound II would serve a similar purpose.

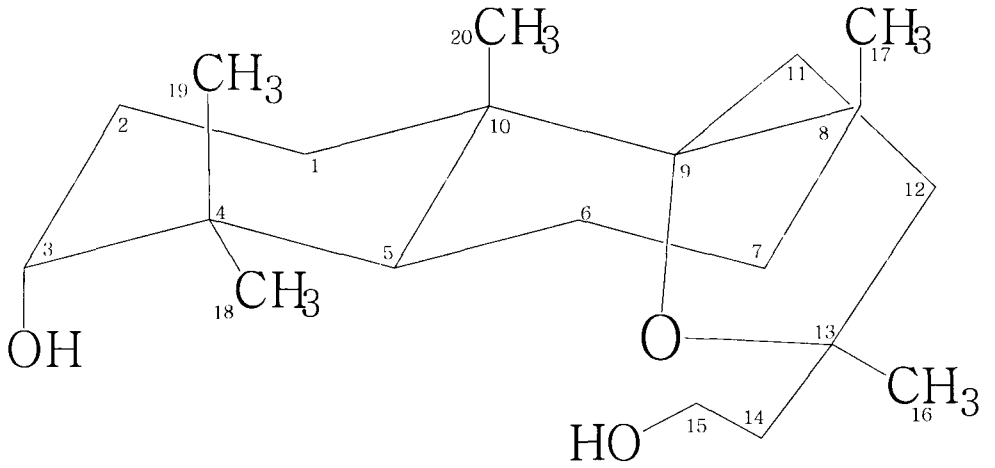

Figure 2.8. Stereochemistry of Compound II.

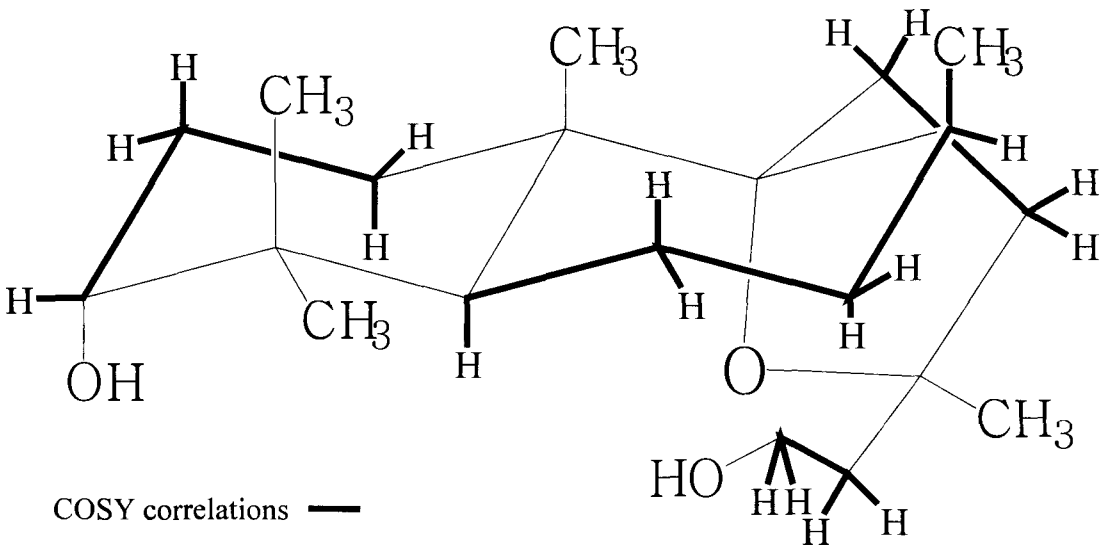

Figure 2.9. COSY correlations for Compound II.

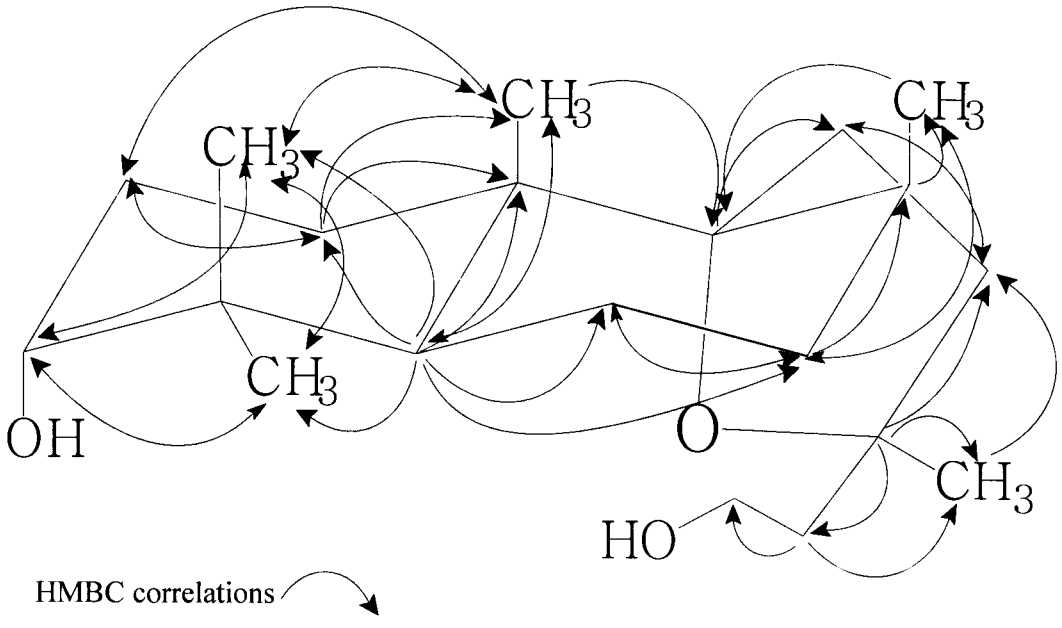

Figure 2.10. HMBC correlations for Compound II.

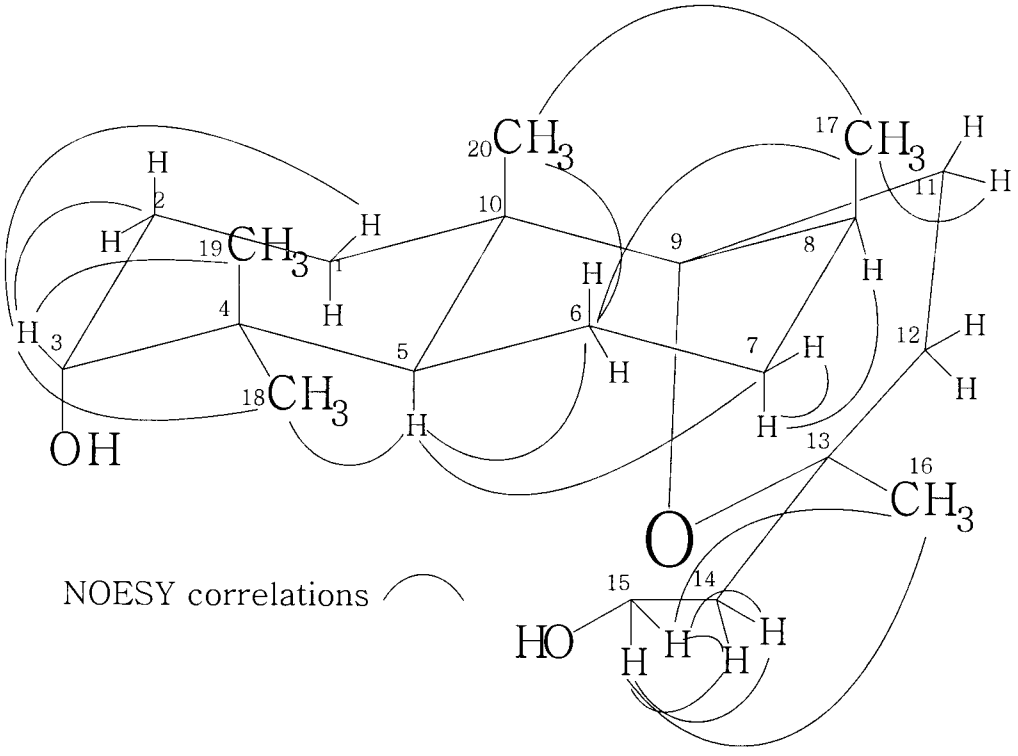

Figure 2.11. NOESY and NOE correlations for Compound II.

2.1.3. p-Bromobenzoyl Derivative of Compound II (Compound IIa): A p-bromobenzoyl derivative of Compound II was synthesized (Figure 2.12), and further NMR experiments on Compound IIa were run. As expected, the chemical shifts of the protons near the benzoate derivative shifted downfield: the C-15 protons shifted from 3.68 and 3.90 ppm, to 3.86 and 3.99 ppm, respectively. Examination of the other protons revealed little change overall; however, there was a slight downfield shift of the axial C-1 proton, and the equatorial C-18 methyl. If Compound II is an epimer at C-13, the p-bromobenzoate derivative would be adjacent spacially to these two groups of protons, and would have an effect on their chemical shifts. Therefore, Compound II may be positively identified as an epimer of Compound I, with an *R* configuration at the C-13 stereocenter.

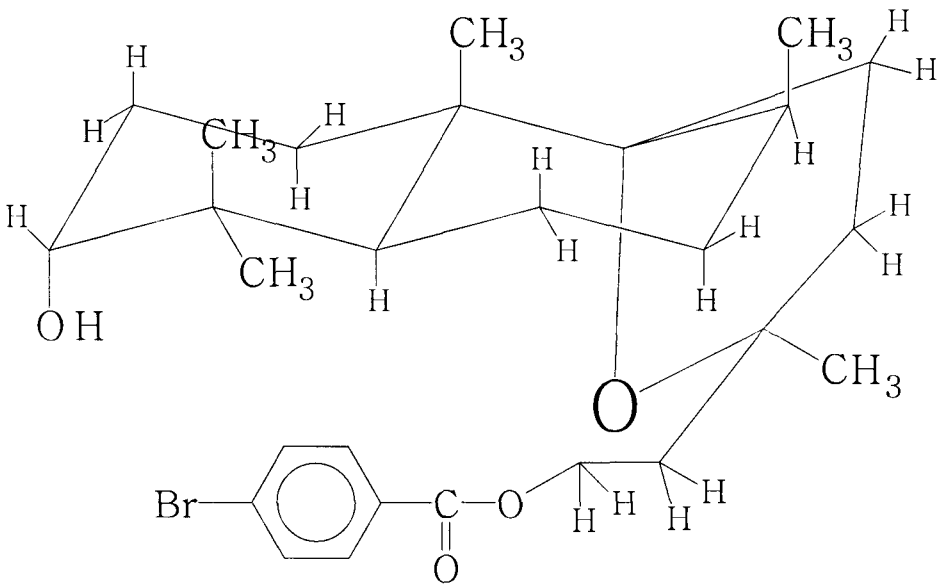

Figure 2.12. Compound IIa, p-bromobenzoate derivative of Compound II..

2.1.4. Diterpene Aldehydes (Compounds IIIa and IIIb): Treadwell had previously isolated and partially characterized Compounds IIIa and b, a pair of unresolved diterpene aldehydes (Figure 1.4) [Treadwell, 1996]. The two aldehydes are likely epimers at C-13, due to biosynthetic considerations and the similarity of NMR spectra to that of

Compound I [Treadwell, 1996]. This hypothesis was confirmed by demonstrating that a mixture of Compounds IIIa and b yielded a mixture of Compounds I and II upon reduction by sodium borohydride, as determined by GC-MS. According to the proposed biosynthetic pathway (Figure 1.2), Compound III is a direct precursor to Compounds I and II. This pathway is supported by the formation of an alcohol in place of the aldehyde at C-15 under laboratory conditions. This result also indicates that Compounds I and II have a common precursor and are produced by the same pathway (Figure 2.13).

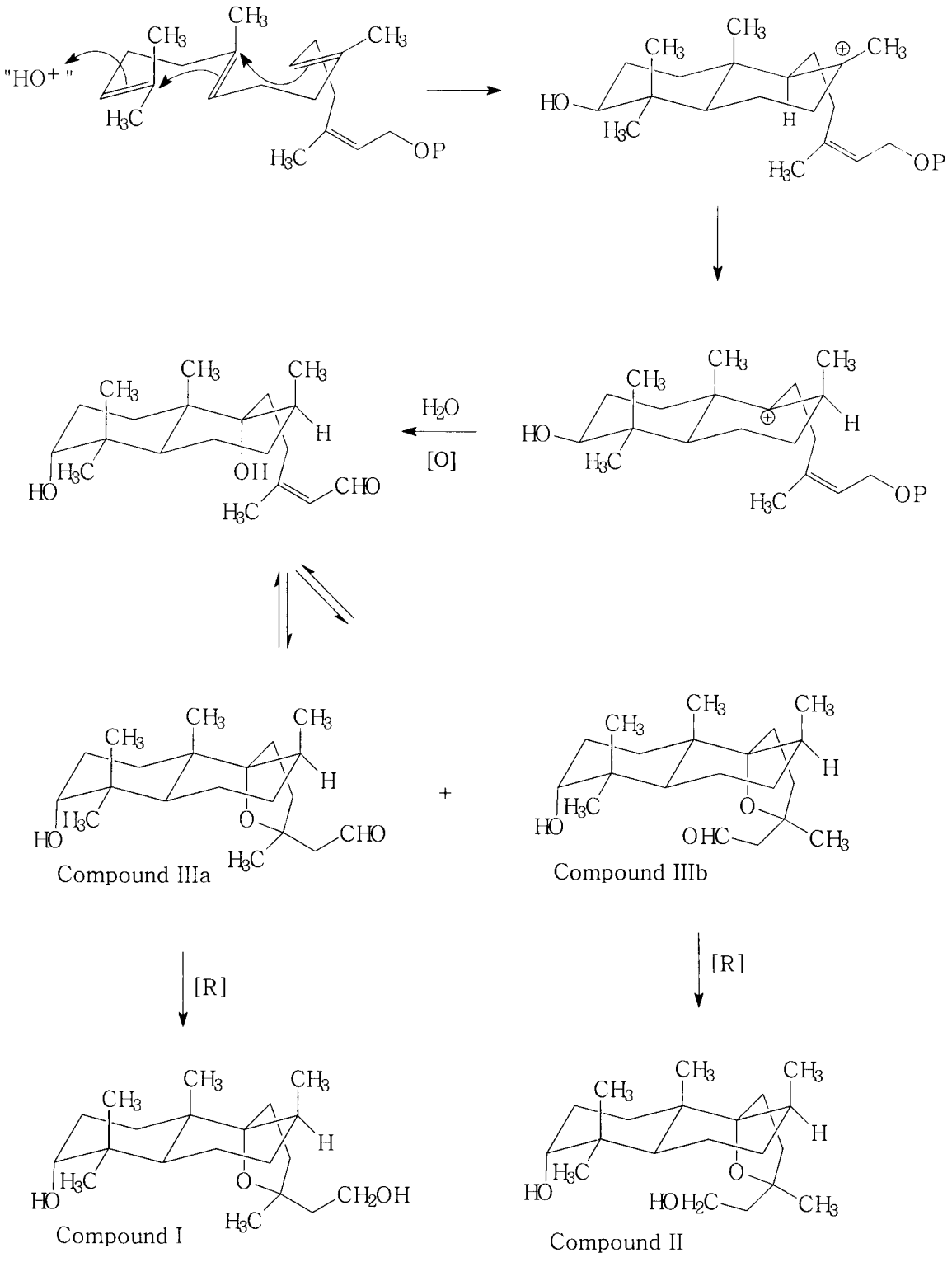

Figure 2.13. Proposed biosynthetic pathway of Compounds I, II, and III.

Table 2.1. Solvent shifts for Compound I. Alpha and beta are relative to C-20.

Mopane Alcohol, $CDCl_3$				Alcohol, d_6-benzene		
ID	Shift ppm	DEPT		ID	Shift ppm	DEPT
1 ^{13}C	26.3	CH_2		1 ^{13}C	26.67	CH_2
1 ^{1}H, alpha	1.05			1 ^{1}H, alpha	0.82	
1 ^{1}H,beta	1.98			1 ^{1}H,beta	2.10	
2 ^{13}C	24.4	CH_2		2 ^{13}C	25.17	CH_2
2 ^{1}H, beta	1.61			2 ^{1}H, beta	1.66	
2 ^{1}H, alpha	1.93			2 ^{1}H, alpha	1.80	
3 ^{13}C	76.1	CH		3 ^{13}C	75.97	CH
3 ^{1}H	3.30			3 ^{1}H	3.34	
4 ^{13}C	37.7	C		4 ^{13}C	37.99	C
5 ^{13}C	41.4	CH		5 ^{13}C	41.42	CH
5 ^{1}H	1.65			5 ^{1}H	1.95	
6 ^{13}C	16.7	CH_2		6 ^{13}C	17.05	CH_2
6 ^{1}H	1.36			6 ^{1}H	1.32	
6 ^{1}H	1.40			6 ^{1}H	1.32	
7 ^{13}C	29.7	CH_2		7 ^{13}C	29.87	CH_2
7 ^{1}H, alpha	1.40			7 ^{1}H, alpha	1.34	
7 ^{1}H, beta	1.98			7 ^{1}H, beta	2.07	
8 ^{13}C	40.4	CH		8 ^{13}C	40.46	CH
8 ^{1}H	1.88			8 ^{1}H	1.75	
9 ^{13}C	94.0	C		9 ^{13}C	93.79	C
10 ^{13}C	41.9	C		10 ^{13}C	42.22	C
11 ^{13}C	29.2	CH_2		11 ^{13}C	29.19	CH_2
11 ^{1}H, *proS*	1.85			11 ^{1}H, *proS*	1.50	
11 ^{1}H, *proR*	2.02			11 ^{1}H, *proR*	1.68	
12 ^{13}C	39.9	CH_2		12 ^{13}C	39.62	CH_2
12 ^{1}H, *proS*	1.73			12 ^{1}H	1.43	
12 ^{1}H, *proR*	1.85			12 ^{1}H	1.43	
13 ^{13}C	84.0	C		13 ^{13}C	83.48	C
14 ^{13}C	42.2	CH_2		14 ^{13}C	42.87	CH_2
14 ^{1}H	1.61			14 ^{1}H	1.30	
14 ^{1}H	2.08			14 ^{1}H	1.91	
15 ^{13}C	60.1	CH_2		15 ^{13}C	59.76	CH_2
15 ^{1}H	3.75			15 ^{1}H	3.71	
15 ^{1}H	4.02			15 ^{1}H	3.96	
16 ^{13}C	26.3	CH_3		16 ^{13}C	28.90	CH_3
16 ^{1}H	1.26			16 ^{1}H	1.04	
17 ^{13}C	18.0	CH_3		17 ^{13}C	18.11	CH_3
17 ^{1}H	1.05			17 ^{1}H	0.90	
18 ^{13}C	28.5	CH_3		18 ^{13}C	26.55	CH_3
18 ^{1}H	1.00			18 ^{1}H	1.03	
19 ^{13}C	22.4	CH_3		19 ^{13}C	22.44	CH_3
19 ^{1}H	0.84			19 ^{1}H	0.75	
20 ^{13}C	18.1	CH_3		20 ^{13}C	18.30	CH_3
20 ^{1}H	0.98			20 ^{1}H	0.80	

Table 2.2. Spectral data for Compound I and Compound Ia, recorded in $CDCl_3$. Data from Bredlie [unpublished data].

Mopane Alcohol Diterpene				Alcohol Diterpene, Benzoated		
ID	Shift ppm	DEPT		ID	Shift ppm	DEPT
1 ^{13}C	26.3	CH_2		1 ^{13}C	26.2	CH_2
1 ^{1}H, alpha	1.05			1 ^{1}H, alpha	1.05	
1 ^{1}H,beta	1.98			1 ^{1}H,beta	2.01	
2 ^{13}C	24.4	CH_2		2 ^{13}C	24.8	CH_2
2 ^{1}H, beta	1.61			2 ^{1}H, beta	1.61	
2 ^{1}H, alpha	1.93			2 ^{1}H, alpha	1.97	
3 ^{13}C	76.1	CH		3 ^{13}C	76.2	CH
3 ^{1}H	3.30			3 ^{1}H	3.38	
4 ^{13}C	37.7	C		4 ^{13}C	40.9	C
5 ^{13}C	41.4	CH		5 ^{13}C	39.2	CH
5 ^{1}H	1.65			5 ^{1}H	1.87	
6 ^{13}C	16.7	CH_2		6 ^{13}C	16.7	CH_2
6 ^{1}H	1.36			6 ^{1}H	1.34	
6 ^{1}H	1.40			6 ^{1}H	1.34	
7 ^{13}C	29.7	CH_2		7 ^{13}C	29.5	CH_2
7 ^{1}H, alpha	1.40			7 ^{1}H, alpha	1.38	
7 ^{1}H, beta	1.98			7 ^{1}H, beta	2.02	
8 ^{13}C	40.4	CH		8 ^{13}C	40.8	CH
8 ^{1}H	1.88			8 ^{1}H	1.96	
9 ^{13}C	94.0	C		9 ^{13}C	92.5	C
10 ^{13}C	41.9	C		10 ^{13}C	41.7	C
11 ^{13}C	29.2	CH_2		11 ^{13}C	28.7	CH_2
11 ^{1}H, *proS*	1.85			11 ^{1}H, *proS*	1.88	
11 ^{1}H, *proR*	2.02			11 ^{1}H, *proR*	1.88	
12 ^{13}C	39.9	CH_2		12 ^{13}C	38.7	CH_2
12 ^{1}H, *proS*	1.73			12 ^{1}H, *proS*	1.74	
12 ^{1}H, *proR*	1.85			12 ^{1}H, *proR*	1.80	
13 ^{13}C	84.0	C		13 ^{13}C	80.8	C
14 ^{13}C	42.2	CH_2		14 ^{13}C	40.6	CH_2
14 ^{1}H	1.61			14 ^{1}H	1.91	
14 ^{1}H	2.08			14 ^{1}H	2.04	
15 ^{13}C	60.1	CH_2		15 ^{13}C	62.9	CH_2
15 ^{1}H	3.75			15 ^{1}H	4.40	
15 ^{1}H	4.02			15 ^{1}H	4.60	
16 ^{13}C	26.3	CH_3		16 ^{13}C	27.5	CH_3
16 ^{1}H	1.26			16 ^{1}H	1.31	
17 ^{13}C	18.0	CH_3		17 ^{13}C	18.2	CH_3
17 ^{1}H	1.05			17 ^{1}H	1.06	
18 ^{13}C	28.5	CH_3		18 ^{13}C	28.5	CH_3
18 ^{1}H	1.00			18 ^{1}H	0.99	
19 ^{13}C	22.4	CH_3		19 ^{13}C	22.2	CH_3
19 ^{1}H	0.84			19 ^{1}H	0.87	
20 ^{13}C	18.1	CH_3		20 ^{13}C	18.2	CH_3
20 ^{1}H	0.98			20 ^{1}H	0.97	

Table 2.3. Spectral data for Compounds I and II, recorded in d_6-benzene.

Mopane Alcohol Epimer				Mopane Alcohol Diterpene		
ID	Shift ppm	DEPT		ID	Shift ppm	DEPT
1 ^{13}C	26.39	CH_2		1 ^{13}C	26.67	CH_2
1 ^{1}H, alpha	0.88			1 ^{1}H, alpha	0.82	
1 ^{1}H,beta	2.12			1 ^{1}H,beta	2.10	
2 ^{13}C	25.36	CH_2		2 ^{13}C	25.17	CH_2
2 ^{1}H, beta	1.59			2 ^{1}H, beta	1.66	
2 ^{1}H, alpha	1.76			2 ^{1}H, alpha	1.80	
3 ^{13}C	75.99	CH		3 ^{13}C	75.97	CH
3 ^{1}H	3.24			3 ^{1}H	3.34	
4 ^{13}C	37.93	C		4 ^{13}C	37.99	C
5 ^{13}C	42.05	CH		5 ^{13}C	41.42	CH
5 ^{1}H	1.99			5 ^{1}H	1.95	
6 ^{13}C	17.12	CH_2		6 ^{13}C	17.05	CH_2
6 ^{1}H	1.31			6 ^{1}H	1.32	
6 ^{1}H	1.31			6 ^{1}H	1.32	
7 ^{13}C	30.02	CH_2		7 ^{13}C	29.87	CH_2
7 ^{1}H, alpha	1.39			7 ^{1}H, alpha	1.34	
7 ^{1}H, beta	2.14			7 ^{1}H, beta	2.07	
8 ^{13}C	38.37	CH		8 ^{13}C	40.46	CH
8 ^{1}H	1.86			8 ^{1}H	1.75	
9 ^{13}C	93.76	C		9 ^{13}C	93.79	C
10 ^{13}C	41.98	C		10 ^{13}C	42.22	C
11 ^{13}C	27.72	CH_2		11 ^{13}C	29.19	CH_2
11 ^{1}H, *proS*	1.54			11 ^{1}H, *proS*	1.50	
11 ^{1}H, *proR*	1.62			11 ^{1}H, *proR*	1.68	
12 ^{13}C	38.96	CH_2		12 ^{13}C	39.62	CH_2
12 ^{1}H, *proS*	1.36			12 ^{1}H	1.43	
12 ^{1}H, *proR*	1.51			12 ^{1}H	1.43	
13 ^{13}C	83.54	C		13 ^{13}C	83.48	C
14 ^{13}C	44.62	CH_2		14 ^{13}C	42.87	CH_2
14 ^{1}H	1.33			14 ^{1}H	1.30	
14 ^{1}H	1.74			14 ^{1}H	1.91	
15 ^{13}C	60.56	CH_2		15 ^{13}C	59.76	CH_2
15 ^{1}H	3.68			15 ^{1}H	3.71	
15 ^{1}H	3.90			15 ^{1}H	3.96	
16 ^{13}C	25.71	CH_3		16 ^{13}C	28.90	CH_3
16 ^{1}H	1.13			16 ^{1}H	1.04	
17 ^{13}C	18.64	CH_3		17 ^{13}C	18.11	CH_3
17 ^{1}H	0.91			17 ^{1}H	0.90	
18 ^{13}C	28.80	CH_3		18 ^{13}C	26.55	CH_3
18 ^{1}H	0.89			18 ^{1}H	1.03	
19 ^{13}C	22.47	CH_3		19 ^{13}C	22.44	CH_3
19 ^{1}H	0.71			19 ^{1}H	0.75	
20 ^{13}C	18.39	CH_3		20 ^{13}C	18.30	CH_3
20 ^{1}H	0.78			20 ^{1}H	0.80	

Table 2.4. Spectral data for Compound II and Compound IIa, recorded in d_6-benzene.*

Mopane Alcohol Epimer				Alcohol Epimer, Benzoated		
ID	Shift ppm	DEPT		ID	Shift ppm	DEPT
1 ^{13}C	26.39	CH_2		1 ^{13}C	26.09	CH_2
1 ^{1}H, alpha	0.88			1 ^{1}H, alpha	0.85	
1 ^{1}H,beta	2.12			1 ^{1}H,beta	2.05	
2 ^{13}C	25.36	CH_2		2 ^{13}C	25.30	CH_2
2 ^{1}H, beta	1.59			2 ^{1}H, beta	1.56	
2 ^{1}H, alpha	1.76			2 ^{1}H, alpha	1.75	
3 ^{13}C	75.99	CH		3 ^{13}C	76.00	CH
3 ^{1}H	3.24			3 ^{1}H	3.20	
4 ^{13}C	37.93	C		4 ^{13}C	42.3	C
5 ^{13}C	42.05	CH		5 ^{13}C	41.92	CH
5 ^{1}H	1.99			5 ^{1}H	1.98	
6 ^{13}C	17.12	CH_2		6 ^{13}C	17.06	CH_2
6 ^{1}H	1.31			6 ^{1}H	1.30	
6 ^{1}H	1.31			6 ^{1}H	1.30	
7 ^{13}C	30.02	CH_2		7 ^{13}C	30.01	CH_2
7 ^{1}H, alpha	1.39			7 ^{1}H, alpha	1.39	
7 ^{1}H, beta	2.14			7 ^{1}H, beta	2.16	
8 ^{13}C	38.37	CH		8 ^{13}C	38.35	CH
8 ^{1}H	1.86			8 ^{1}H	1.85	
9 ^{13}C	93.76	C		9 ^{13}C	93.43	C
10 ^{13}C	41.98	C		10 ^{13}C	42.0	C
11 ^{13}C	27.72	CH_2		11 ^{13}C	25.11	CH_2
11 ^{1}H, *proS*	1.54			11 ^{1}H, *proS*	1.51	
11 ^{1}H, *proR*	1.62			11 ^{1}H, *proR*	1.70	
12 ^{13}C	38.96	CH_2		12 ^{13}C	38.14	CH_2
12 ^{1}H, *proS*	1.36			12 ^{1}H, *proS*	1.47	
12 ^{1}H, *proR*	1.51			12 ^{1}H, *proR*	1.51	
13 ^{13}C	83.54	C		13 ^{13}C	83.31	C
14 ^{13}C	44.62	CH_2		14 ^{13}C	44.47	CH_2
14 ^{1}H	1.33			14 ^{1}H	1.29	
14 ^{1}H	1.74			14 ^{1}H	1.72	
15 ^{13}C	60.56	CH_2		15 ^{13}C	60.09	CH_2
15 ^{1}H	3.68			15 ^{1}H	3.86	
15 ^{1}H	3.90			15 ^{1}H	3.99	
16 ^{13}C	25.71	CH_3		16 ^{13}C	25.59	CH_3
16 ^{1}H	1.13			16 ^{1}H	1.11	
17 ^{13}C	18.64	CH_3		17 ^{13}C	18.57	CH_3
17 ^{1}H	0.91			17 ^{1}H	0.91	
18 ^{13}C	28.80	CH_3		18 ^{13}C	28.65	CH_3
18 ^{1}H	0.89			18 ^{1}H	0.84	
19 ^{13}C	22.47	CH_3		19 ^{13}C	22.43	CH_3
19 ^{1}H	0.71			19 ^{1}H	0.70	
20 ^{13}C	18.39	CH_3		20 ^{13}C	18.29	CH_3
20 ^{1}H	0.78			20 ^{1}H	0.78	

*Chemical shifts for carbons in IIa were indirectly recorded using HSQC and HMBC, as the quantity was too low to obtain ^{13}C NMR data directly.

2.2. Growth Study

2.2.1. Plant Growth Patterns: In all age classes, the control group, or "unrinsed" seedlings, were smaller than the treatment group, or "rinsed" seedlings (Table 2.5). In almost all cases, this difference was statistically significant. Among the root samples, two age classes were not statistically significant at the 90% confidence level: the 15 week samples and the 19 week samples. In both of these cases, the standard deviations of the average weights were very large: greater that half of the dry weight. Similarly, one of the shoot age classes failed the t-test: the 19 week samples. In this case, however, the 19-week-old rinsed samples had a slightly smaller average weight than the 17-week-old rinsed samples. This is not the case for the unrinsed samples: the 19-week age class is much larger than the 17-week age class.

Table 2.5. Average Dry Weights of Plant Samples.

Root Samples, Rinsed			Root Samples, Unrinsed			T-test	
Age (weeks)	Ave. Dry Wt. (mg)	SD (mg)	Age (weeks)	Ave. Dry Wt. (mg)	SD (mg)	t_{calc}	$t_{calc} > t^*_{90\%, 6\ df}$?
7	53.2	10.8	7	37.4	2.7	3.5	yes
9	58.3	8.4	9	39.5	4.4	4.8	yes
10	170.8	27.3	10	98.3	15.3	5.7	yes
13	177.1	42.9	13	97.7	39.3	3.3	yes
15	197.8	126.6	15	114.6	40.9	1.5	no
17	365.5	124.7	17	207.0	88.0	2.5	yes
19	377.9	78.3	19	329.0	150.1	0.7	no
Shoot Samples, Rinsed			Shoot Samples, Unrinsed			T-test	
Age (weeks)	Ave. Dry Wt. (mg)	SD (mg)	Age (weeks)	Ave. Dry Wt. (mg)	SD (mg)	t_{calc}	$t_{calc} > t^*_{90\%, 6\ df}$?
7	336.2	21.0	7	299.4	39.8	2.0	yes
9	353.1	35.1	9	305.6	47.1	2.0	yes
10	597.9	67.8	10	488.0	91.9	2.4	yes
13	529.0	136.8	13	340.2	153.0	2.3	yes
15	741.5	106.9	15	383.0	76.5	6.7	yes
17	924.6	205.0	17	469.1	93.5	5.0	yes
19	850.0	86.0	19	779.0	110.7	1.0	no

This evidence alone suggests that the seedlings are not able to utilize the terpenes found on the seed coat for primary metabolic processes, or at least that reabsorption of terpenes

are not a determining factor in seed germination and growth rates. In fact, removing the resin from the seed coat prior to germination surprisingly resulted in a greater average weight of both the above-ground portion of the plant, the "shoot," and the below-ground plant material, or "root."

C. mopane has an exceptionally high root biomass that well exceeds the leaf biomass, as well as a decisively shallow root system. There are indications that the root:leaf ratio exceeds that of several other vegetation types. According to one study, the root:leaf ratio of mopane averages 16.95, based on root dry mass and leaf dry mass of 10 excavation sites. In comparison, secondary forest has a root:leaf ratio of 0.71, and mature corn is 1.15 [Smit and Rethman, 1998].

These root:leaf ratios were not observed in this growth study. Average root:leaf ratios for 2 month old unrinsed seedlings were 0.141 and 0.164 for rinsed seedlings; 5 month old seedlings were 0.462 and 0.466, respectively. This large discrepancy from the Smit study may have been caused by several factors. Most studies focus on mature plants, which may have a much different pattern in root:leaf growth ratios than seedlings. This argument is supported by the increase in root:leaf ratio as the seedlings age. The plants in this study were grown in greenhouse conditions in pots, which may stunt root growth, especially since these plants have an ectomycorrhizzal relationship with fungal hyphae in their native environment. These observed root:leaf ratios may be skewed by collection techniques in this study as well: no special effort was made to collect all the fine root hairs in the plant samples, so the root weights may be biased low, although not enough to explain the discrepancy.

The combination of a shallow root system and high root biomass implies a high potential for severe competition with herbaceous plants. However, while they are somewhat susceptible to competition, especially by grasses, mopane usually occurs in monotypic stands [Timberlake, 1995]. This may be due in part to chemicals exuded by the roots, or

allelopathy. It is possible that mopane seedlings exude allelopathic chemicals at concentrations high enough to inhibit competition from other plants, at the risk of slowing their own growth. Removing these terpenes, as in the rinsed seeds, may allow for faster growth by the seedlings. The unrinsed seeds must still contend with high doses of allelopathic chemicals, and their growth may be stunted relative to the rinsed group. Alternatively, it is possible that the seed resins coating the seeds act as a physical barrier, and hinder the absorption of water and nutrients, thus slowing the growth of the unrinsed seeds relative the the rinsed group.

Special care was utilized in this study to avoid damaging the plants before harvest, to prevent any possible trigger for induced defenses. There does not seem to be many studies of inducible defenses in *C. mopane*; however, it has been shown that physical damage does not increase the likelihood of fungal attacks in adult trees [Smith and Shah-Smith, 1999]. Whether this was due to production of chemical defenses was not investigated.

2.2.2. GC-MS Results: Three sesquiterpenes associated with allelopathy were identified in the root extracts by GC-MS: α-copaene, α-cubebene, and trans-caryophyllene. Two in particular, α-copaene and trans-caryophyllene, represented the majority of the crude extract in the roots and shoots (Figure 2.14, 2.15). These compounds have also been found in the resin coating the seeds [Treadwell, 1996], and in this study in almost all the samples, through all the age classes.

Two other groups of terpenes were found in large quantities in these samples: the diterpene alcohol Compound I, and the pair of diterpene aldehydes Compounds IIIa and IIIb. Unlike the sesquiterpenes, these diterpenes were not found in the younger age classes of plant samples, and Compound I appeared later than Compounds III a and b. This is consistent with the proposed biosynthesis of these compounds (Figure 2.13). Interesting, Compound II was not observed in this growth study. During preliminary

studies, Compounds I and II were found in equal amounts in extracts of several different plant materials. In particular, Compound II was not found in the seed extracts, and was present in very low quantities in the leaf extracts (Figure 2.16).

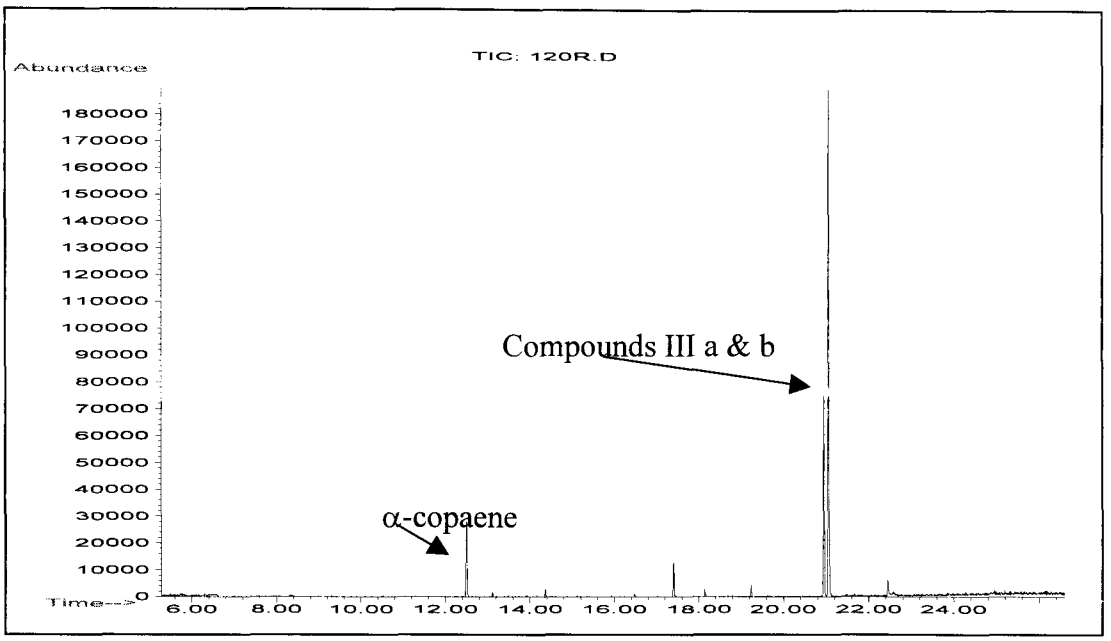

Figure 2.14. GC trace of a typical root sample, sample #120R.

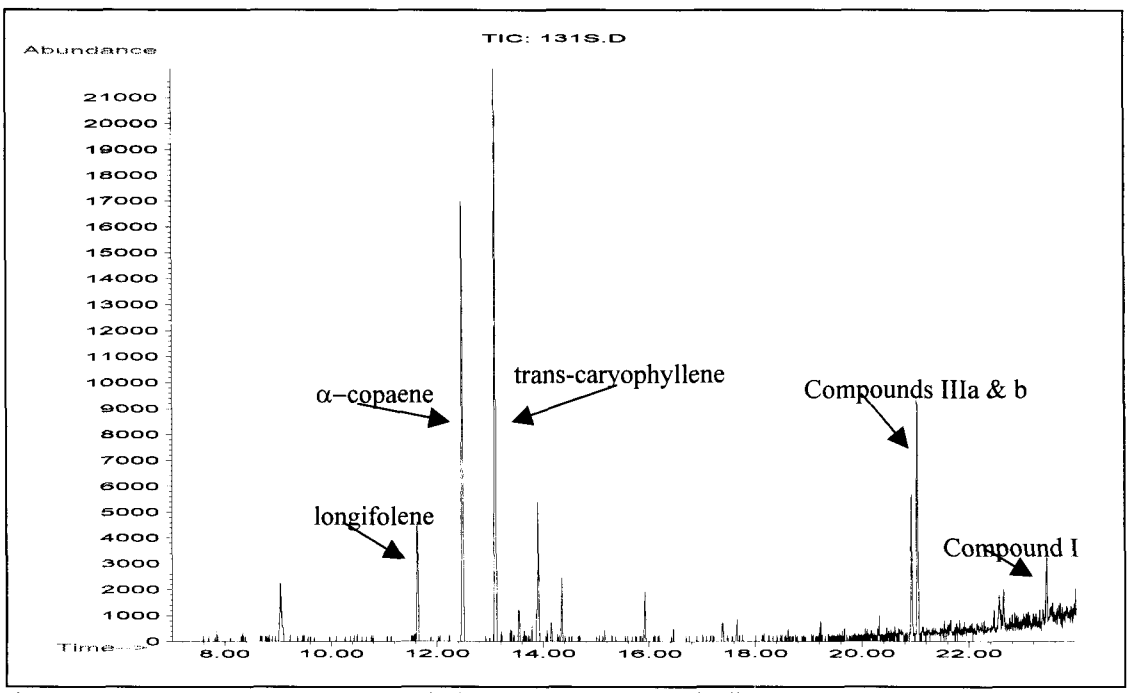

Figure 2.15. GC trace of a typical shoot sample, sample #131S.

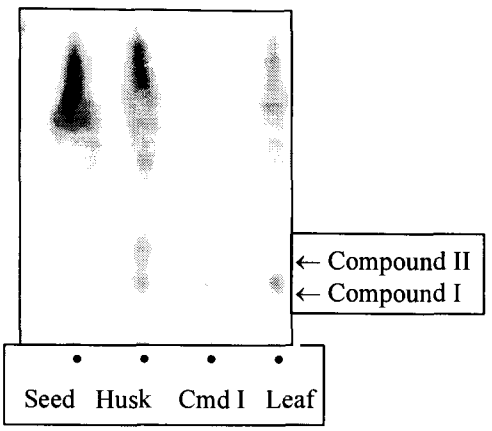

Figure 2.16. Comparative amounts of Compounds I and II in seed, seed husk, and leaf extracts. TLC plate eluted with 10% $CHCl_3$ in Et_2O.

The concentrations of alpha-copaene and trans-caryophyllene, in almost all cases, were higher in the plants grown from rinsed seeds than the plants grown from unrinsed seeds (Tables 2.6-2.7). This held true for both root and shoot samples. Similarly, the concentrations of Compound I and Compounds IIIa and IIIb were found to be higher in rinsed samples than in the control samples (Tables 2.8-2.9).

To determine if the concentrations of terpenes statistically differed between treatments, t-tests were performed for each sample type (root or shoot) and age class. Although almost all age classes had higher concentrations of the measured terpenes in rinsed samples, fewer than half were upheld by t-tests. This is most likely due to the very large variability observed among the individual samples, and seen in the large standard deviations of terpene concentration.

Table 2.6. Concentrations of terpenes in root and shoot extracts. Units in ppm per gram plant material.

	Root, Rinsed		Root, Not Rinsed		Statistics	
Age (weeks)	[α-copaene]	S.D.	[α-copaene]	S.D.	t_{calc}	$t_{calc} > t^*_{80\%, 6\ df}$?
7	61.0	53.44	16.8	13.43	1.96	yes
9	43.0	26.12	31.4	24.62	0.80	no
10	60.8	42.41	55.3	30.57	0.26	no
13	66.2	40.20	69.3	29.85	0.15	no
15	171	163.94	88.7	85.88	1.09	no
17	105	48.44	78.2	27.06	1.18	no
19	64.7	35.60	52.8	15.90	0.75	no
	Shoot, Rinsed		Shoot, Not Rinsed		Statistics	
Age (weeks)	[α-copaene]	S.D.	[α-copaene]	S.D.	t_{calc}	$t_{calc} > t^*_{80\%, 6\ df}$?
7	2.60	1.93	1.15	0.79	1.70	yes
9	8.29	8.87	3.66	3.15	1.20	no
10	7.03	2.53	9.76	4.25	1.35	no
13	16.2	7.42	10.6	5.62	1.46	yes
15	11.4	2.90	8.14	2.28	2.22	yes
17	12.5	8.65	10.6	2.76	0.52	no
19	17.5	5.76	10.6	9.29	1.54	yes

Table 2.7. Concentrations of trans-caryophyllene. Units in ppm per gram plant material.

	Root, Rinsed		Root, Not Rinsed		Statistics	
Age (weeks)	[t-caryo-phyllene]	S.D.	[t-caryo-phyllene]	S.D.	t_{calc}	$t_{calc} > t^*_{80\%, 6\ df}$?
7	2.21	3.78	0.31	0.48	1.22	no
9	11.56	25.48	2.62	3.38	0.85	no
10	3.51	6.90	10.31	5.68	1.87	yes
13	6.78	8.92	0.00	0.00	1.86	yes
15	5.78	8.98	4.66	9.95	0.21	no
17	8.67	6.10	2.79	3.13	2.10	yes
19	6.53	3.70	1.75	3.69	2.24	yes
	Shoot, Rinsed		Shoot, Not Rinsed		Statistics	
Age (weeks)	[t-caryo-phyllene]	S.D.	[t-caryo-phyllene]	S.D.	t_{calc}	$t_{calc} > t^*_{80\%, 6\ df}$?
7	4.20	2.27	3.86	2.77	0.23	no
9	3.19	1.14	1.97	1.65	1.50	yes
10	5.76	5.72	5.60	5.20	0.05	no
13	14.09	13.95	5.02	3.63	1.54	yes
15	16.85	13.84	12.73	9.37	0.60	no
17	4.88	3.57	4.11	1.66	0.48	no
19	9.23	4.00	8.41	3.61	0.37	no

Table 2.8. Concentrations of diterpene aldehyde mixture. Units in ppm per gram plant material.

	Root, Rinsed		Root, Not Rinsed		Statistics	
Age (weeks)	[alde-hyde]	S.D.	[alde-hyde]	S.D.	t_{calc}	$t_{calc} > t^*_{80\%, 6\ df}$?
7	134.00	81.95	35.39	28.39	2.79	yes
9	102.37	77.97	39.84	44.45	1.71	yes
10	243.43	123.78	9.80	8.51	4.61	yes
13	113.64	65.84	100.26	29.79	0.45	no
15	666.74	596.39	727.46	852.88	0.14	no
17	298.53	89.37	258.89	145.70	0.57	no
19	194.26	69.96	186.37	97.92	0.16	no
	Shoot, Rinsed		Shoot, Not Rinsed		Statistics	
Age (weeks)	[alde-hyde]	S.D.	[alde-hyde]	S.D.	t_{calc}	$t_{calc} > t^*_{80\%, 6\ df}$?
7	0.00	0.00	0.00	0.00	0.00	no
9	1.55	1.06	0.81	1.57	0.95	no
10	2.46	2.06	1.90	2.09	0.47	no
13	3.53	1.51	1.37	1.24	2.71	yes
15	6.65	4.23	5.90	5.81	0.26	no
17	6.43	3.76	2.92	2.19	1.98	yes
19	7.24	3.81	7.14	3.69	0.05	no

Table 2.9. Concentrations of diterpene alcohol. Units in ppm per gram plant material.

	Root, Rinsed		Root, Not Rinsed		Statistics	
Age (weeks)	[alcohol]	S.D.	[alcohol]	S.D.	t_{calc}	$t_{calc} > t^*_{80\%, 6\ df}$?
7	0.00	0.00	0.00	0.00	0.00	no
9	0.00	0.00	0.00	0.00	0.00	no
10	1.83	2.84	0.00	0.00	1.58	yes
13	3.12	2.55	0.00	0.00	3.00	yes
15	6.84	2.23	0.00	0.00	7.52	yes
17	3.63	1.75	6.44	8.31	0.81	no
19	2.49	2.85	2.08	2.01	0.29	no
	Shoot, Rinsed		Shoot, Not Rinsed		Statistics	
Age (weeks)	[alcohol]	S.D.	[alcohol]	S.D.	t_{calc}	$t_{calc} > t^*_{80\%, 6\ df}$?
7	0.00	0.00	0.00	0.00	0.00	no
9	0.00	0.00	0.00	0.00	0.00	no
10	0.00	0.00	0.00	0.00	0.00	no
13	0.93	0.93	0.00	0.00	2.45	yes
15	1.73	0.43	0.14	0.33	7.18	yes
17	1.55	0.47	1.74	0.56	0.62	no
19	1.65	0.31	1.56	0.37	0.44	no

Simple univariate statistics do not seem to be adequate to examine this data set, and ANOVA is more appropriate for comparing three or more variables. ANOVA also assumes that there are no time trends, and this growth study experiment was designed to collect samples at discrete intervals. More useful information may be garnered from a multivariate statistics approach, like principle component analysis (PCA). PCA is a pattern recognition tool that allows for visualization of hidden structures and correlations in a data set. It can be used to filter noise from data sets, and can indicate the amount of variation contained by each measurement variable (Beebe, 1998). PCA can look at time trends in a data set.

When PCA is performed on the root data, two clusters appear on the scores plot (Figure 2.17). The scores plot shows the relationship of the sample to the axes defined by the principle components, and reveals how the samples are related to each other given the measurements that have been made (Beebe, 1998). Samples that are close to each other are similar with respect to the total amount of variation, and show chemical similarity. The clustering observed indicates that there is some pattern within the data, and that two different chemical systems are acting on the samples. In Figure 2.17, the upper cluster consists of rinsed samples, and the lower cluster is unrinsed samples. Therefore, by simple visual examination of the PCA scores plot, it is obvious that there is a real difference in treatment effect of the root samples. This pattern is also seen when the shoot samples are examined by PCA (Figure 2.19). In Figure 2.19, the upper cluster consists of rinsed samples, and the lower cluster represents unrinsed samples.

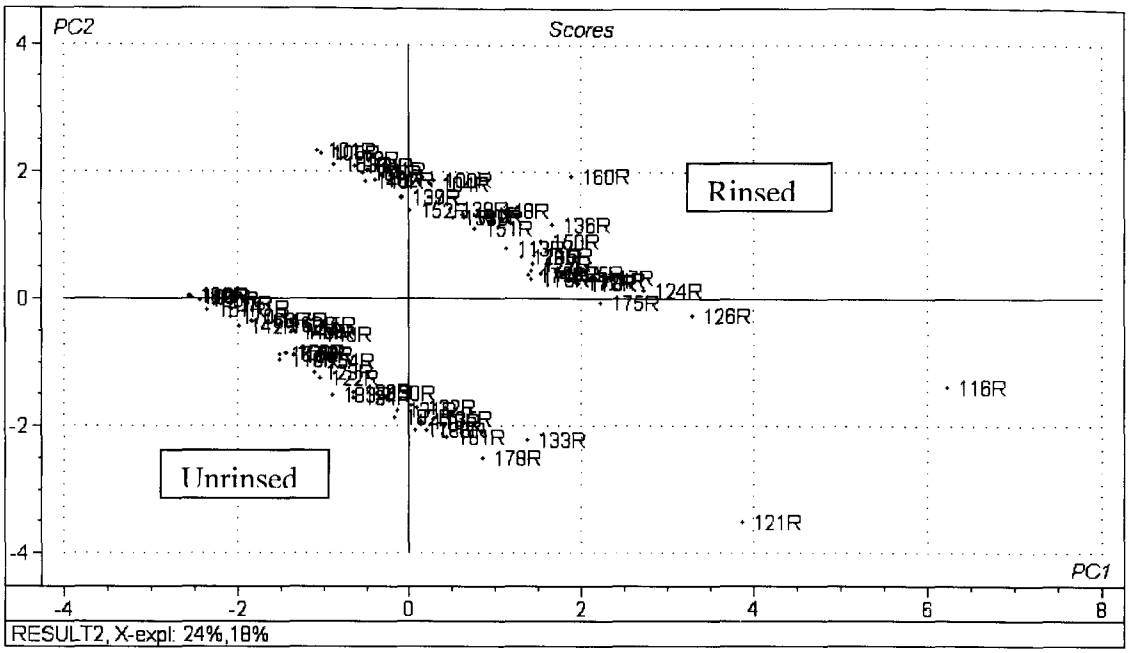

Figure 2.17. PCA scores plot of root samples.

The X-loadings plots determine which variables are important for describing the variation in the original data set. The loadings are the cosine of the angle between the principle component and the original variables, and describe how the original measurement variables are related to each of the new principle component axes. For the root samples, all of the measurement variables, including concentration of terpenes, age, and weight, are positively correlated to the categorical variable Rinse Yes (Figure 2.18). This indicates that samples that are rinsed have a higher mass and higher concentrations of α-copaene, t-caryophyllene, Compound I, and Compounds IIIa and b than samples that were not rinsed. Again, this pattern is repeated when the shoot samples are examined (Figure 2.20). Rinsed samples are positively correlated with concentrations of terpenes, age, and weight.

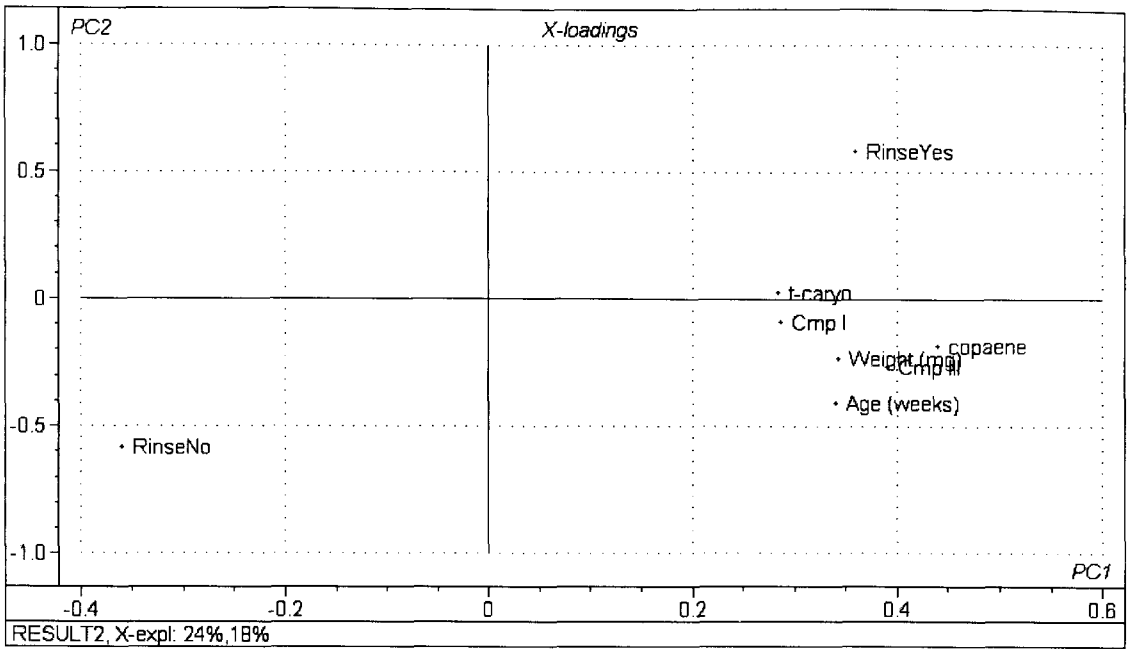

Figure 2.18. PCA X-loadings plot for root samples.

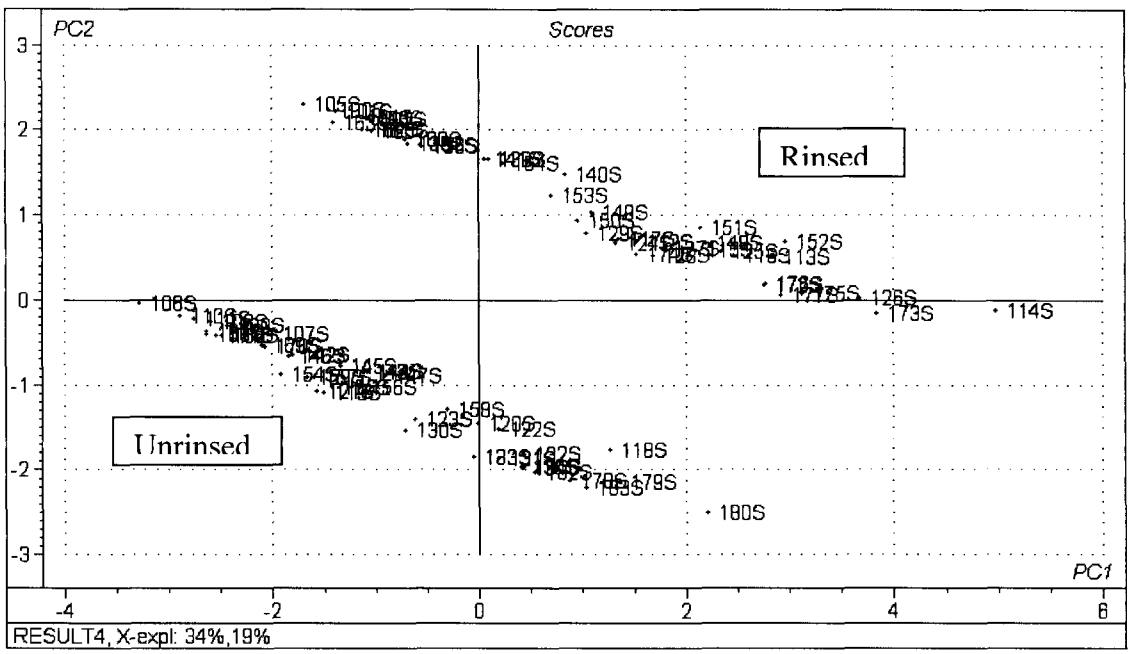

Figure 2.19. PCA scores plot of shoot samples.

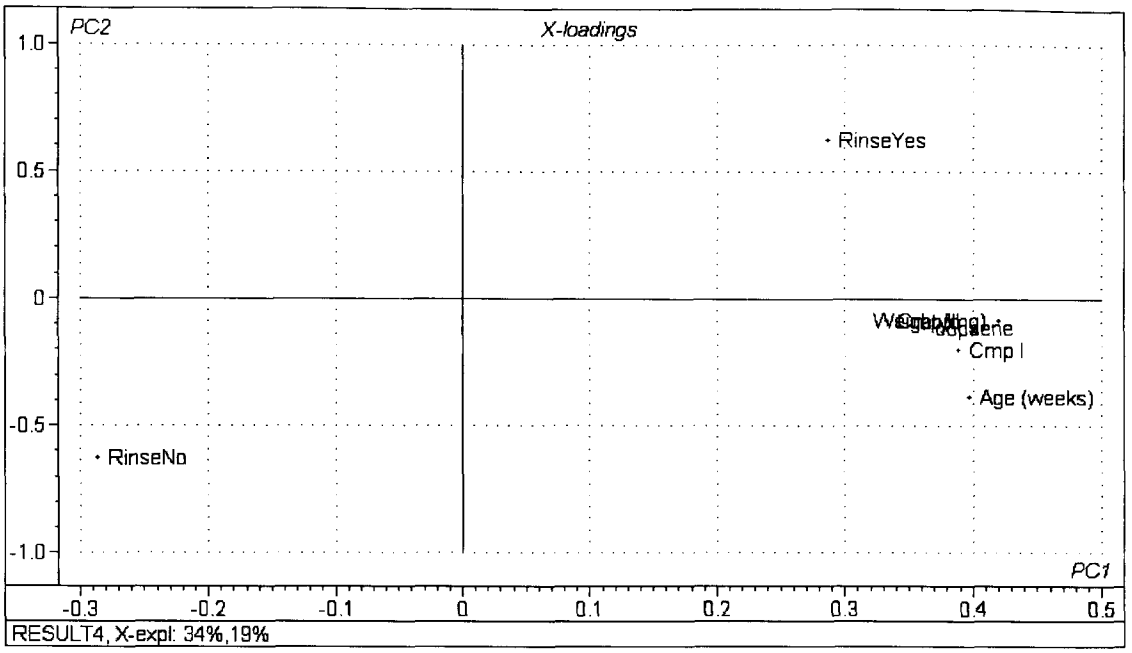

Figure 2.20. PCA X-loadings plot for shoot samples.

While t-tests did not prove a treatment difference unambiguously for all age classes, almost half showed that rinsed samples have statistically higher levels of terpenes and a greater mass than control samples. Multivariate statistical analysis shows that there is a real difference in root and shoot samples, and that the difference is due to treatment of the seeds prior to germination. In both cases, rinsed samples had higher levels of all four terpenes, and higher weights than unrinsed samples. By using two different statistical approaches, real trends in the data are much more likely to be seen and verified.

3.0. CONCLUSIONS

Labdane diterpenes are an important class of natural products to study due to their variety of biological activities. Many labdanes have been found to have antibacterial, antifungal, anti-inflammatory, cytotoxic, and enzymatic inhibition effects [Singh *et al*, 1998]. The diterpenes in this study had previously shown strong antibacterial activity [Treadwell, 1996], and the low incidence of herbivory on *Colophospermum mopane* indicates the possibility of other activities.

The diterpenes identified in this study represent an early stage in the biogenesis of 9,13-epoxylabdanes. Most of labdanes are highly substituted and oxygenated, while the diterpenes in this study are, relatively speaking, more primitive. These diterpenes have a close biosynthetic relationship to geranylgeranyl pyrophosphate, and may be considered a "missing link." All of the known 9,13-epoxylabdanes are highly oxygenated, and their stereochemistry is inconsistent with the simple predictions found in Figures 1.2 and 2.13. While the literature of many labdanes reported prior to the development of modern NMR techniques is often contradictory and sometimes suspect, it remains clear that the labdanes reported in this study relatively simple compounds.

The growth study experiment yielded some surprising results. Chemically removing the resin coat on the seeds prior to germination caused more rapid growth and terpene production than control plants. This result seems to indicate that seedlings cannot utilize the terpenes on the seed for raw materials for primary production, and that chemically defending the seed with terpenes is an expensive proposition for the plant. Removal of the resin promotes growth and production of terpenes, most likely in one of two ways. It is possible that the resin, in addition to protection against herbivory, may also help to preserve the seed. The seeds may lay around for years, before natural conditions allow for germination. The resin may help prevent the seed from drying out during drought years. If this is true, the resin would exclude water and nutrients from penetrating the seed, and thus rinsed seeds would grow more quickly than unrinsed

seeds. Alternatively, it is possible that such a large concentration of terpenes is found in the seed coat that it approaches auto-toxic levels. While the mopane seedlings are hindered and grow more slowly than they would without the resin, other plants are completely inhibited, and cannot compete with mopane seedlings for light, water, and nutrients. Removing the resin eliminates a poisoning effect, and the rinsed seeds can grow more quickly than controls. Any delay that occurs during early growth stages can have long-lasting effects on the growth and development of a seedling.

There are several avenues for further study. There are still components in the extracts of root, leaf, seed, and seed coat that have not been isolated in a quantity great enough for analysis, and a complete study of the constituents may yield further interesting compounds.

Secondly, further biological assays should be done, on both crude extracts and the isolated diterpenes. These should be assayed for anti-insecticidal deterrence. This is especially important because with wild silk moth larvae *Gonimbrasia belina* feeds exclusively on mopane leaves, and represents a vital indigenous food source.

Finally, throughout the course of the growth study, the entire plant was harvested, to avoid triggering any induced defensive mechanisms. Since it has been observed that elephants are predisposed to feed and batter the same stands of mopane, it appears that there is either no induction of defense through herbivory, or else that the loss of plant matter beyond a certain extent causes a decrease in secondary product production. It is worthwhile to see if this pattern is replicated in juvenile mopane seedlings. It would also be interesting to see if the resin removed from the seeds can actually inhibit germination of the seeds of other species of plants. This may answer the question of whether rinsing mopane seeds removes a toxic effect, or removes a physical barrier.

4.0. EXPERIMENTAL

4.1. Terpene Identification

4.1.1. Sample collection: Leaves were collected by Dr. Joseph Dudley from trees in the Sengwa Wildlife Research Area, located within the Sebungwe region of Zimbabwe (between 18°01' to 18°13' S, 28°03' to 28°20' E). Young "post-flush" and mature leaves were collected in 1996, air-dried for 48 hours, then placed overnight in an herbarium drying oven [Dudley, 1998]. Mopane seed samples were collected by Dr. Joseph Dudley in January of 1996 at Hwange National Park in Zimbabwe and air-dried.

4.1.2. Extraction and Isolation: The seed husks were removed from *C. mopane* seeds, then pulverized in a Waring blender. The husks (86.5 g) were extracted and re-extracted in hexanes (OmniSolv non UV reagent). The combined extracts were filtered and evaporated under reduced pressure to yield 3.16 g of yellow oil. A solvent system for separation by column chromatography was determined by using TLC plates (EM Separations Technology, Silica Gel 60 F_{254}) developed with 5% H_2SO_4 in EtOH.

The crude extract was flash-chromatographed through silica gel (Baker, 40 μm) in two portions using two solvent systems: first 10% EtOAc (VWR Reagent, ACS) in $CHCl_3$ (VWR Reagent, ACS), then 10% $CHCl_3$ in EtOAc. Twenty mL fractions were collected from each column. Fractions 39-45 of the first column and fractions 36-46 of the second were combined, yielding 33.7 mg of impure Compound I. This was rechromatographed using a gradient from 10%, 20%, 40%, and 100% Et_2O (VWR Reagent, ACS) in $CHCl_3$, collecting 5 mL fractions. Fractions 7-13 were combined, yielding 32 mg of Compound I; however, traces of impurity remained. This was chromatographed again in a two-solvent system, using 30% Et_2O in $CHCl_3$ and 80% Et_2O in $CHCl_3$, yielding 25 mg of pure Compound I. Impure Compound II was found in fractions 24-30 and 22-26 of the original flash chromatography column of the crude extract. These fractions were combined and rechromatographed using a gradient from 100% $CHCl_3$ to 10%, 20%, 40%, and 100% Et_2O in $CHCl_3$, which yielded 71 mg of impure Compound II. This was

again rechromatographed using a gradient from 10%, 30%, and 50% Et_2O in $CHCl_3$. Final yield of purified Compound II was 17 mg.

Compounds III a and b were isolated from the hexane root extracts from the growth study experiment. In many cases, the major component of the root extract were Compounds III a and b (Figure 2.14), so minimal clean-up chromatography was required. The root extracts were collected and evaporated under reduced pressure to yield approximately 100 mg of crude material. This was flash-chromatographed through silica gel (Baker, 40 μm) using a solvent gradient from 100%, 85%, 70%, and 50% $CHCl_3$ in Et_2O. Fractions four through seven were recombined to yield 14 mg of Compounds III a and b, with some trace impurities.

4.1.3. NMR Spectroscopy: All spectra were recorded with a 300 MHz Varian Mercury Spectrometer. Spectra were obtained in either d-chloroform (Aldrich, 99.8 atom % D) or in d_6-benzene (Aldrich, 99.6 atom % D). Tetramethylsilane (TMS) was not used; spectra were instead referenced to the solvent peak.

4.1.4. p-Brombenzoyl Derivative of Compound II: As per the method of Treadwell [1996], 15.7 mg of pure Compound II (0.0485 mmol) was dissolved in 1.0 mL of dry pyridine freshly distilled over CaH_2 in a 10 mL round bottom flask. To this, 23.8 mg (0.110 mmol) of p-bromobenzoyl chloride (Aldrich, 98%) was added drop wise. The flask was fitted with a reflux condenser and the solution was stirred for 60 minutes at 90° C. The solution was poured into 10 mL of cold Et_2O and washed with two 10 mL portions of cold 10% HCl. The combined acid layers were back-extracted with two 15 mL portions of Et_2O. The combined organic layers were washed twice with 20 mL portions of saturated $NaHCO_3$ solution, and then dried over $MgSO_4$. Concentration under reduced pressure yielded 25.9 mg of crude product. TLC analysis revealed the presence of impurities, primarily unreacted Compound II and p-bromobenzoic acid. The crude

product was flash-chromatographed using 20% Et_2O in $CHCl_3$. Fractions 5-7 were combined to yield 12.0 mg of pure Compound IIa.

4.1.5. Reduction of Compounds III a and b: Seven milligrams of partially-purified Compounds III a and b were reduced using $NaBH_4$ in methanol. Following the procedure in *Microscale Organic Laboratory* [Mayo *et al*, 1994], a stock reducing solution was prepared using 51.2 mg of anhydrous $NaOCH_3$ dissolved in 5 mL of methanol. While stirring with a magnetic stir-bar, 101.3 mg of $NaBH_4$ was added. This solution was stirred vigorously for several minutes on a stir-plate. A 100 μL aliquot of the stock reducing solution (0.02 M) was added drop-wise to 7 mg of Compound III dissolved in 2 mL methanol (0.01 M). This was allowed to react with stirring in the fume hood, and the reaction was followed by TLC at 30 minute intervals. After two hours, the aldehydes had not been appreciably reduced, so another 500 μL aliquot of the stock reducing solution was added. After an additional two hours, no reaction was evident. A 1 mL aliquot of the reducing solution was added to the reaction mixture, and within 30 minutes, TLC showed the formation of two spots consistent with Compounds I and II. After 1 hour, no Compound III remained. GC-MS confirmed an absence of Compound III, and showed two peaks with retention times and fragmentation patterns consistent with Compounds I and II. The molecular ion peak of 324 was not present; however, M^+ was often absent in prior mass spectra if the samples were not concentrated.

This reaction required reducing agent far in excess of the amount predicted, probably due to the age and diminished reactivity of the $NaOCH_3$ and $NaBH_4$ used. The reaction mixture also contained a much lower amount of aldehyde (2×10^{-5} mol) than the published procedure recommended (1×10^{-3} mol).

4.1.6. Molecular Modeling: All molecular modeling was performed on HyperChem™ Molecular Modeling System Release 6.02 for Windows, copyright © 2000 Hypercube, Inc. For both Compounds I and II, the initial configuration of the trans-decalin ring was

chair-chair, chair-boat, boat-chair, or boat-boat. Energies were minimized by two semi-empirical methods (AM1 and PM3) and by a molecular modeling method (MM+).

4.2. Growth Study

4.2.1. Seed Preparation and Growth: Two groups of plants were studied: those grown from untreated seed, and those grown from seeds whose terpenes had been removed by rinsing with an organic solvent. In both cases, the seed husk was removed from the seeds. Seeds ranged in weight from 0.19 g to 0.55 g, with an average seed weight of 0.36 g for both groups. Unrinsed seeds, as the control group is labeled, were planted into cell-packs in a sterile seed-starting mix composed mainly of vermiculite and sphagnum peat moss. They were placed on a heating pad to raise the soil temperature to 25° C. Germination took place in approximately 5 days. After the first true leaf appeared, the seedlings were transplanted into a potting soil mix (ProMix). Ambient temperatures ranged from 15-24° C. The seedlings received a balanced fertilizer at quarter-strength with every watering (60 ppm N, P, K).

The seeds in the rinsed group were removed from the seed husk, then soaked in a triple volume of hexanes for 60 minutes to extract terpenes from resin ducts located on the seed coat. Due to the corrugated surface of C. mopane seeds, it was impossible to physically remove the seed coat and the resin ducts; therefore, the resin was extracted chemically. Tests showed that the seeds soaked in hexanes up to 5 hours suffered no reduction in germination; however, extraction efficiency did not improve more than 2% after 1 hour. After soaking, the seeds were drained, then rinsed with another portion of hexanes. The seeds were allowed to air-dry for several hours to remove residual solvent, then planted in the same fashion as the unrinsed group. Germination for the rinsed group also occurred in approximately 5 days.

4.2.2. Sample Collection and Extraction: Starting at 7 weeks of age, 6 seedlings were collected from each group. Further collections were made at 9, 10, 13, 15, 17, and 19

weeks. In all cases, the entire plant was harvested, to avoid the possible induction of defensive chemicals. The above-ground portion of the plant, the "shoot" in this study, was clipped off at soil level, and cut into approximately one-inch sections. The soil prior to harvest was allowed to dry slightly, to facilitate removal of the root matter. Any clinging dirt was carefully brushed off the root material, but not rinsed, to prevent possible loss of terpenes, and the roots were then cut into one-inch sections. Each sample was placed into a plastic bag, labeled, and lyophilized for 24-36 hours until dry. The samples were then weighed, partially crushed, and extracted for 48 hours. The entire shoot samples were extracted with 15 mL hexanes, and the entire root samples were extracted in 10 mL hexanes. In all cases this volume was sufficient to completely cover the plant material.

After 48 hours, the extracts were filtered through Whatman 1 Qualitative filter paper, and the plant matter was rinsed with an additional 2 mL hexanes. These samples were then diluted in hexanes, and analyzed by GC-MS.

4.2.3. GC-MS: The instrument used in this study was a Hewlett Packard 5890 Series II Gas Chromatograph with a 5972 Series Mass Selective Detector. All samples were analyzed using method EMILY3.M (Table 4.1). Concentrations of analyte were determined from a calibration curve of the sesquiterpene longifolene in ethanol (Figure 4.1). These ten standards ranged in concentration from 0.0208 to 0.416 ppm. The leaf and root extracts were diluted to fall within the range of the calibration curve.

Table 4.1. GC-MS Conditions for EMILY3.M.

Column	EC-5 (5% phenyl, 95% methyl silicone), 30 m x 0.25 mm ID
	0.25 μm film thickness
Carrier gas	ultra high purity helium
Autosampler	Hewlett Packard 5890 Front Tray Autosampler.
Injection volume	1.0 μL
Injection port temp	275° C
Detector temp	300° C
Inlet pressure	8.3 psi
Flow	1.00 mL/min
Oven temp	70° C
	Hold 3.00 minutes
	Ramp 10°/min to 280°
	hold 4.00 min
MS mode	Total Ion Current (TIC), 100-400 amu

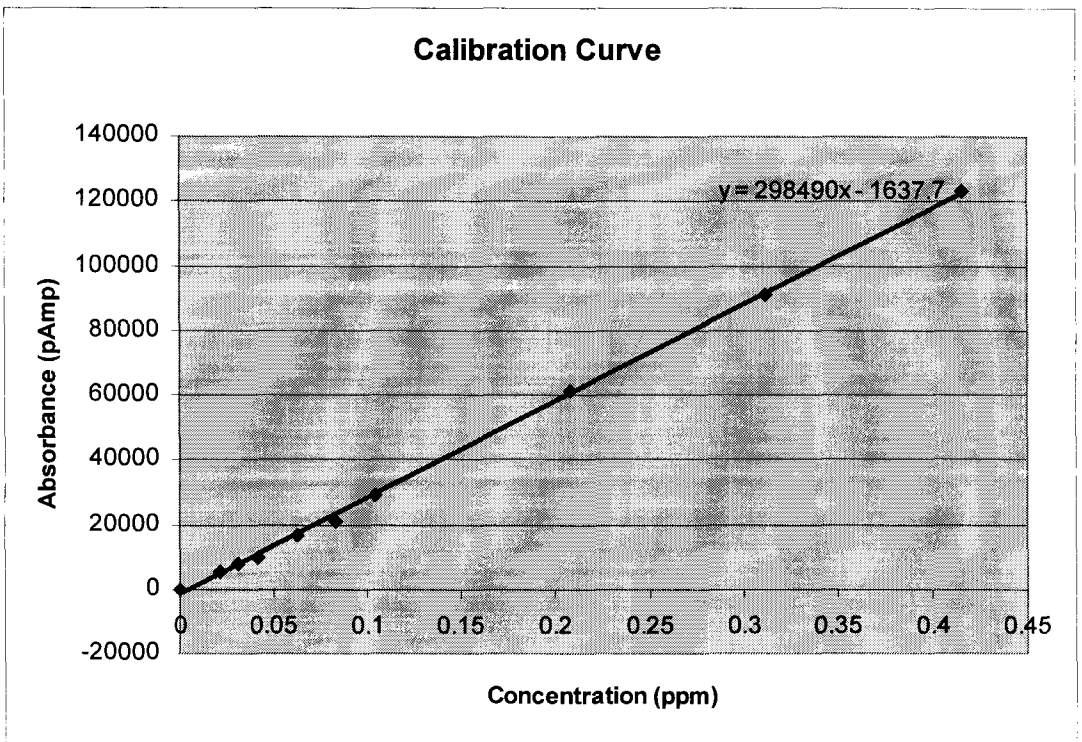

Figure 4.1. Calibration curve, longifolene in ethanol.

4.2.4. Multivariate Analysis: Multivariate analysis was performed using The Unscrambler® 6.11a software for Windows, copyright 1997 CAMO ASA, Oslo, Norway. This software allowed for a multivariate statistical analysis of the concentrations of terpenes and plant size and age with the sample description variables. Variables included dry weight of sample and sample age (Table C.1), and concentrations of α-copaene, t-caryophyllene, Compound I, and combined Compounds IIIa and b (Table C.2). Treatment type were used as categorical values, and were coded as matrices of +1 or 0 (rinsed or not rinsed).

5.0. REFERENCES

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APPENDIX A. HYPERCHEM MOLECULAR MODELING

Table A.1. Calculated minimum energies for Compounds I and II, relative to lowest-energy chair-chair configuration, using HyperChem™. Semi-emperical methods include AM1 and PM3. Molecular modeling methods include MM+. All energies are in kcal/mol.

		Starting Configuration			
Compound	Method	Chair-Chair*	Chair-Boat**	Boat-Chair**	Boat-Boat**
I	AM1	(-5664.46)	+1.82	+3.13	+3.78
I	PM3	(-5661.77)	+5.95	+10.34	+16.46
I	MM+	(+49.87)	+6.08	+8.15	+11.02
II	AM1	(-5663.57)	-2.30	+2.85	+2.96
II	PM3	(-5661.50)	+4.16	+10.56	+12.25
II	MM+	(+50.89)	+12.69	+8.08	+13.75

*Actual calculated minimum energies.

**Minimum energies relative to chair-chair configuration.

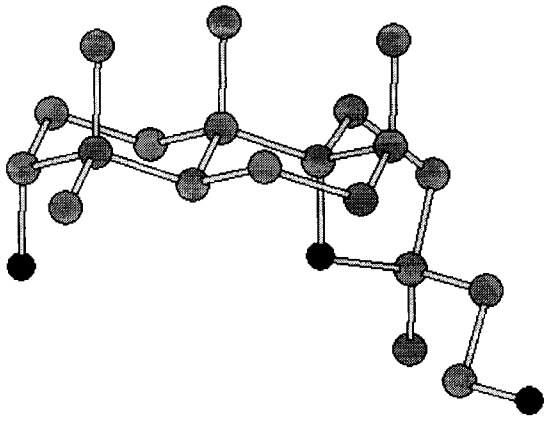


Figure A.1. Compound I, initial chair-chair conformation, prior to geometry optimization.

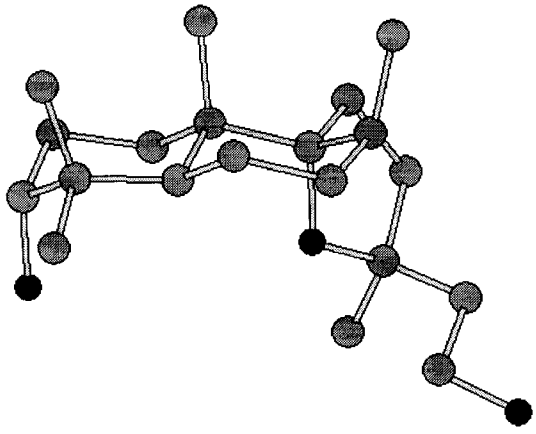


Figure A.2. Compound I, chair-chair conformation. Lowest predicted energy conformation, optimized by semi-empirical method PM3.

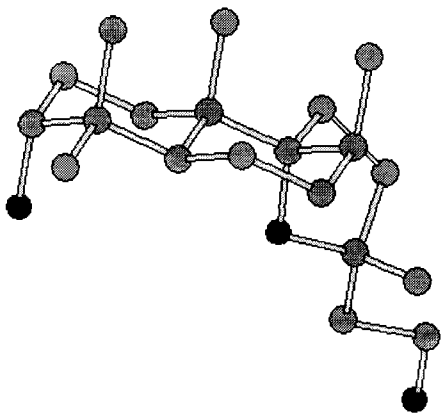


Figure A.3. Compound II, initial chair-chair conformation, prior to geometry optimization.

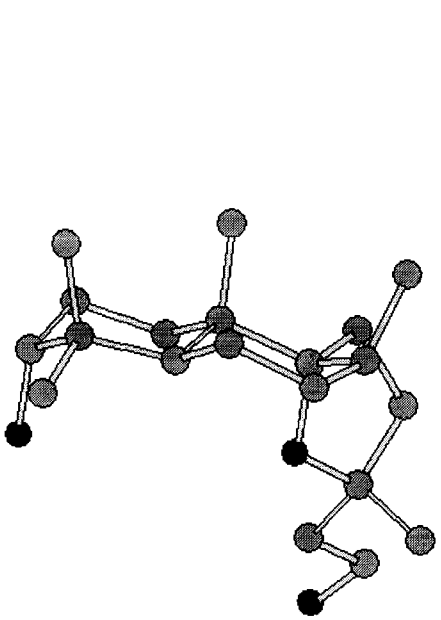


Figure A.4. Compound II, chair-chair conformation. Lowest predicted energy conformation, optimized by semi-empirical method PM3.

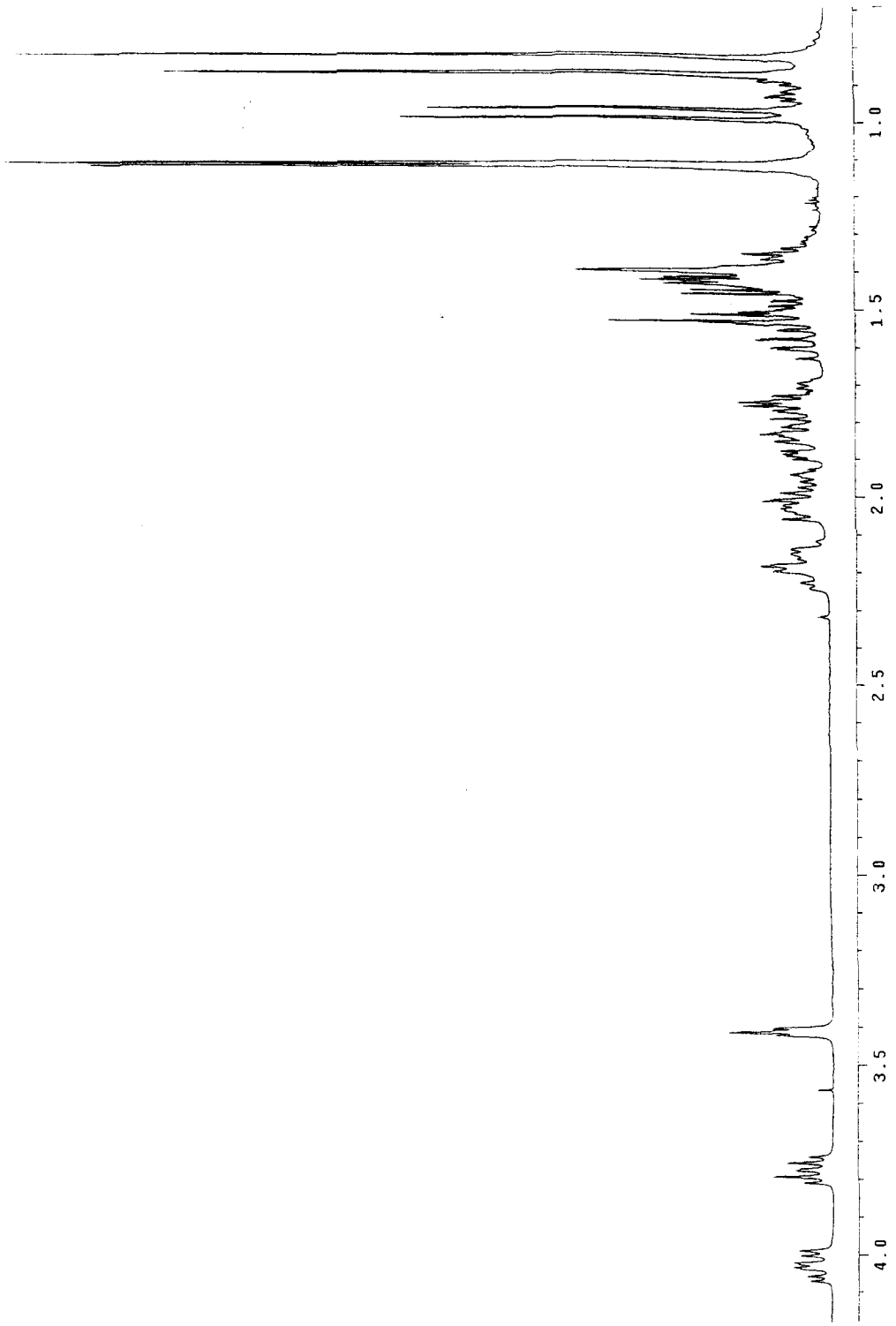


Figure B.1. ^{1}H spectrum of Compound I, in d_6-benzene.

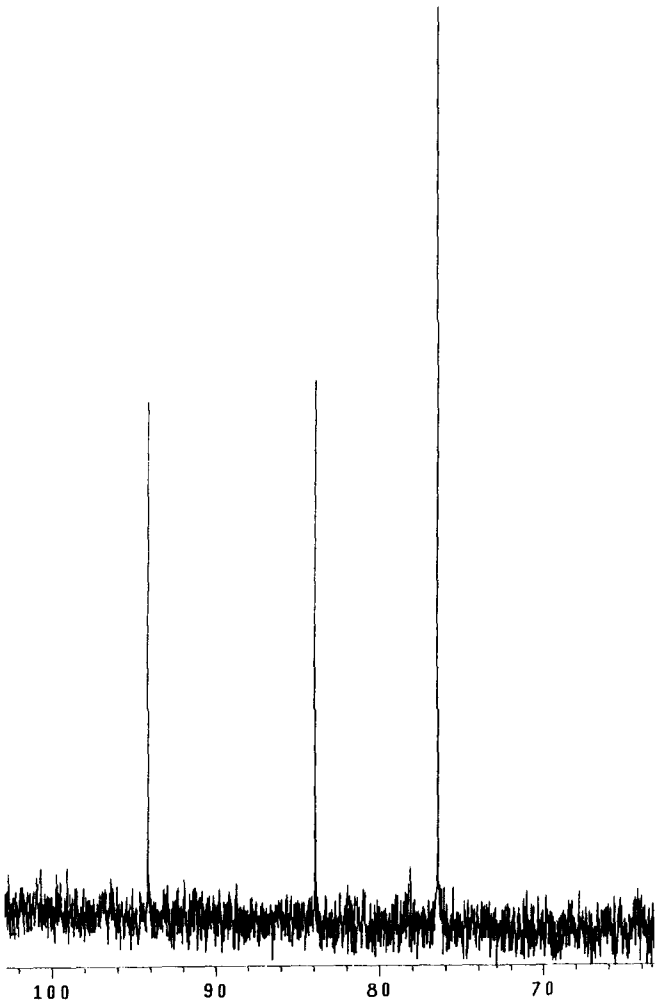
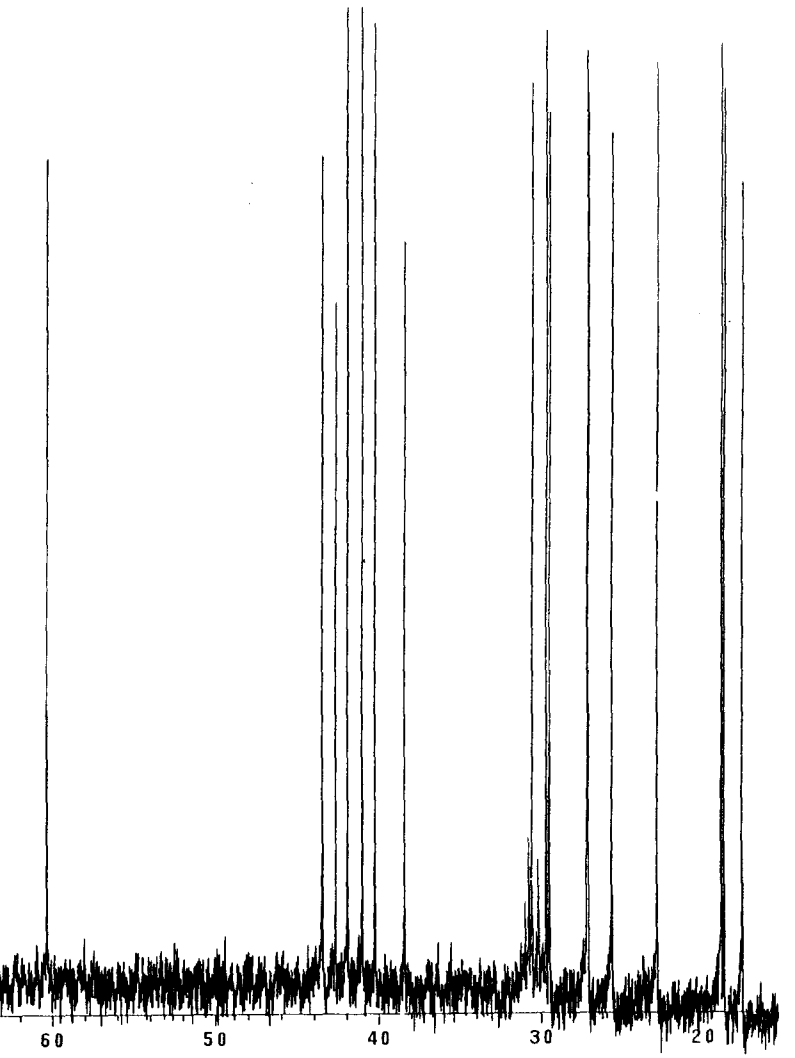


Figure B.2. ^{13}C spectrum of Compound I, in d_6-benzene.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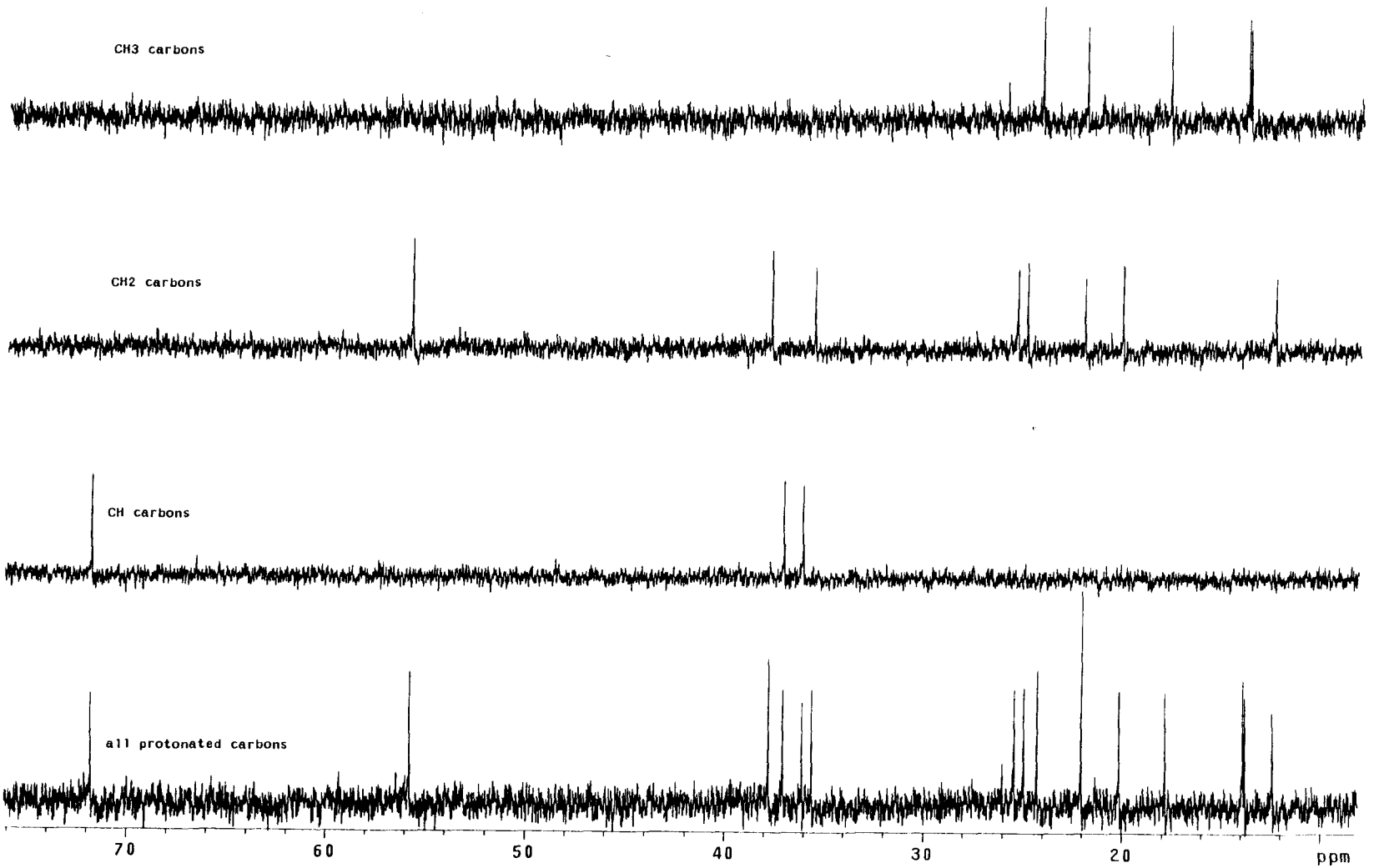


Figure B.3. DEPT spectrum of Compound I, in d_6-benzene.

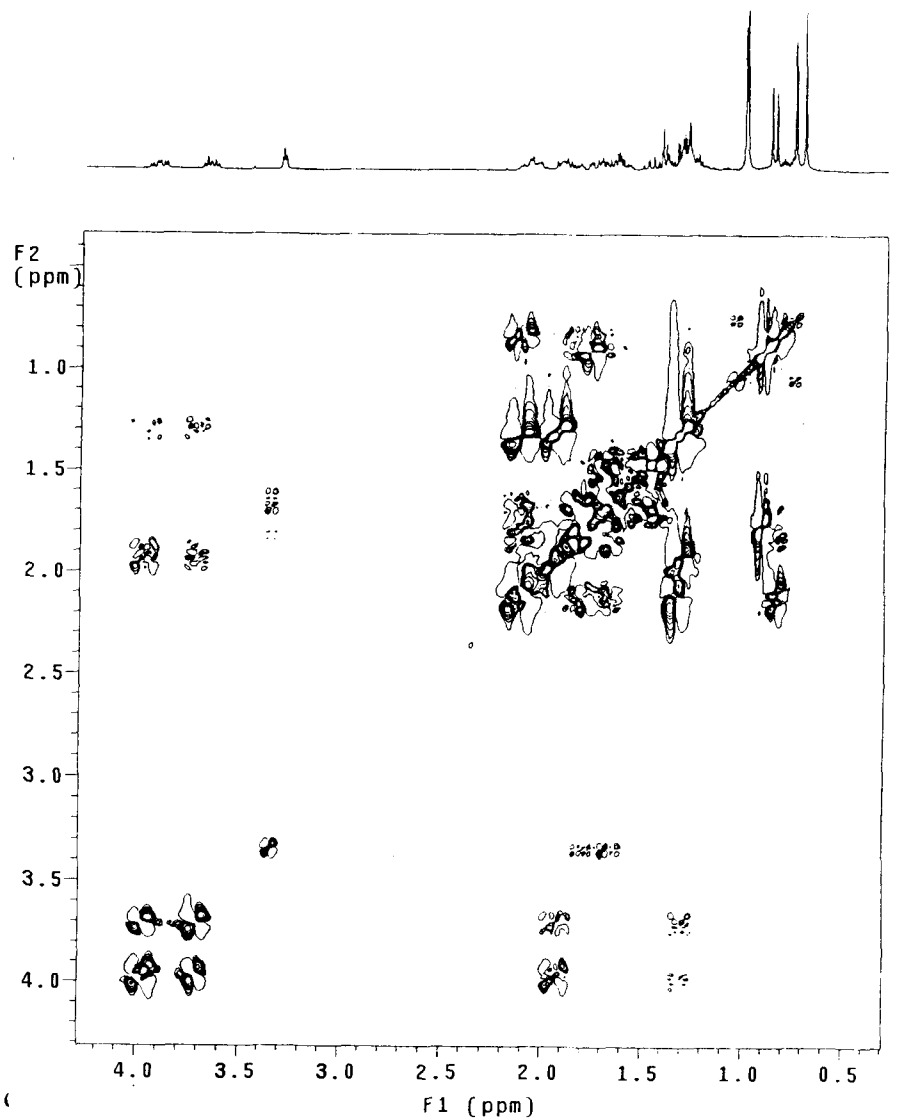


Figure B.4. gDQCOSY spectrum of Compound I, in (

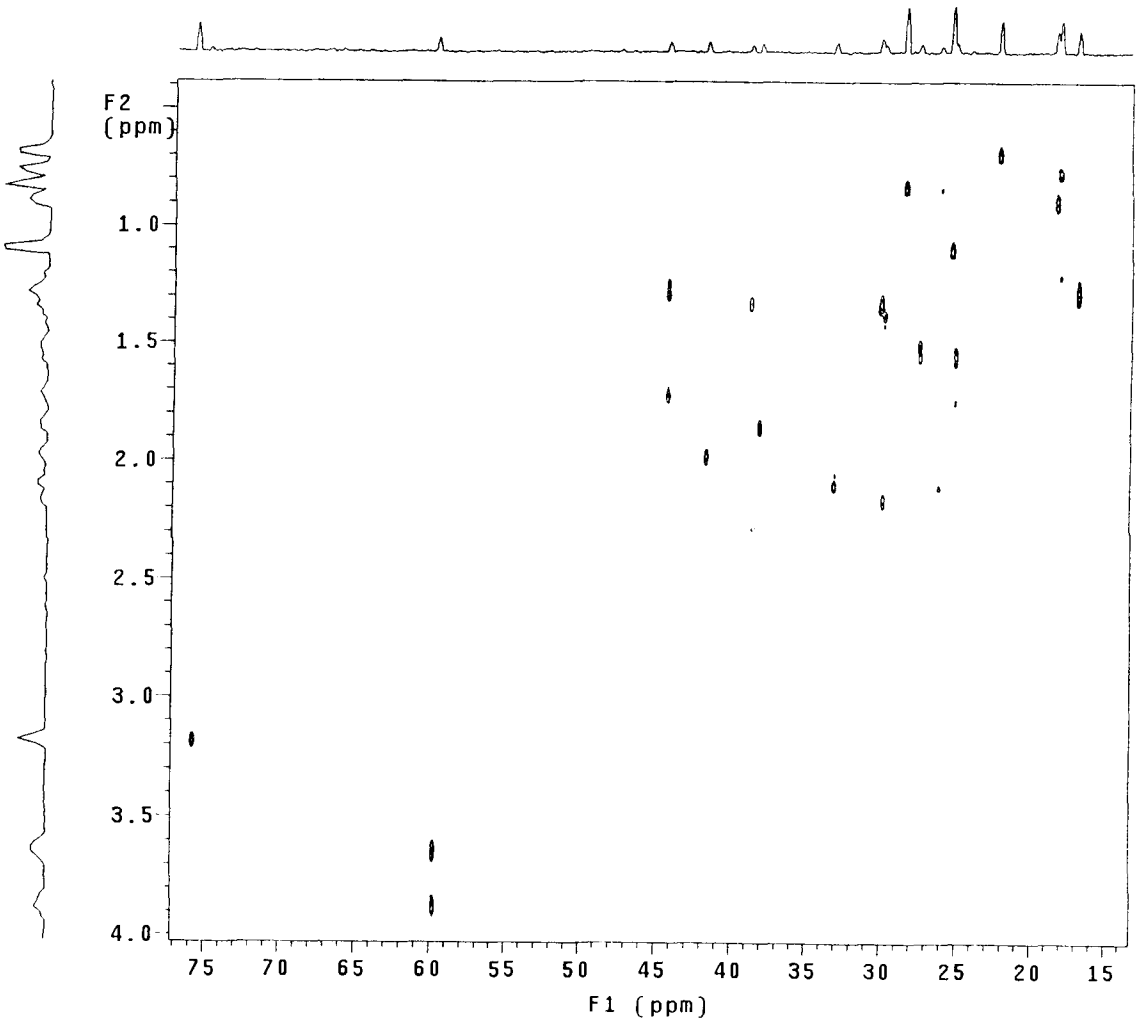


Figure B.5. HSQC spectrum of Compound I, in d_6-benzene.

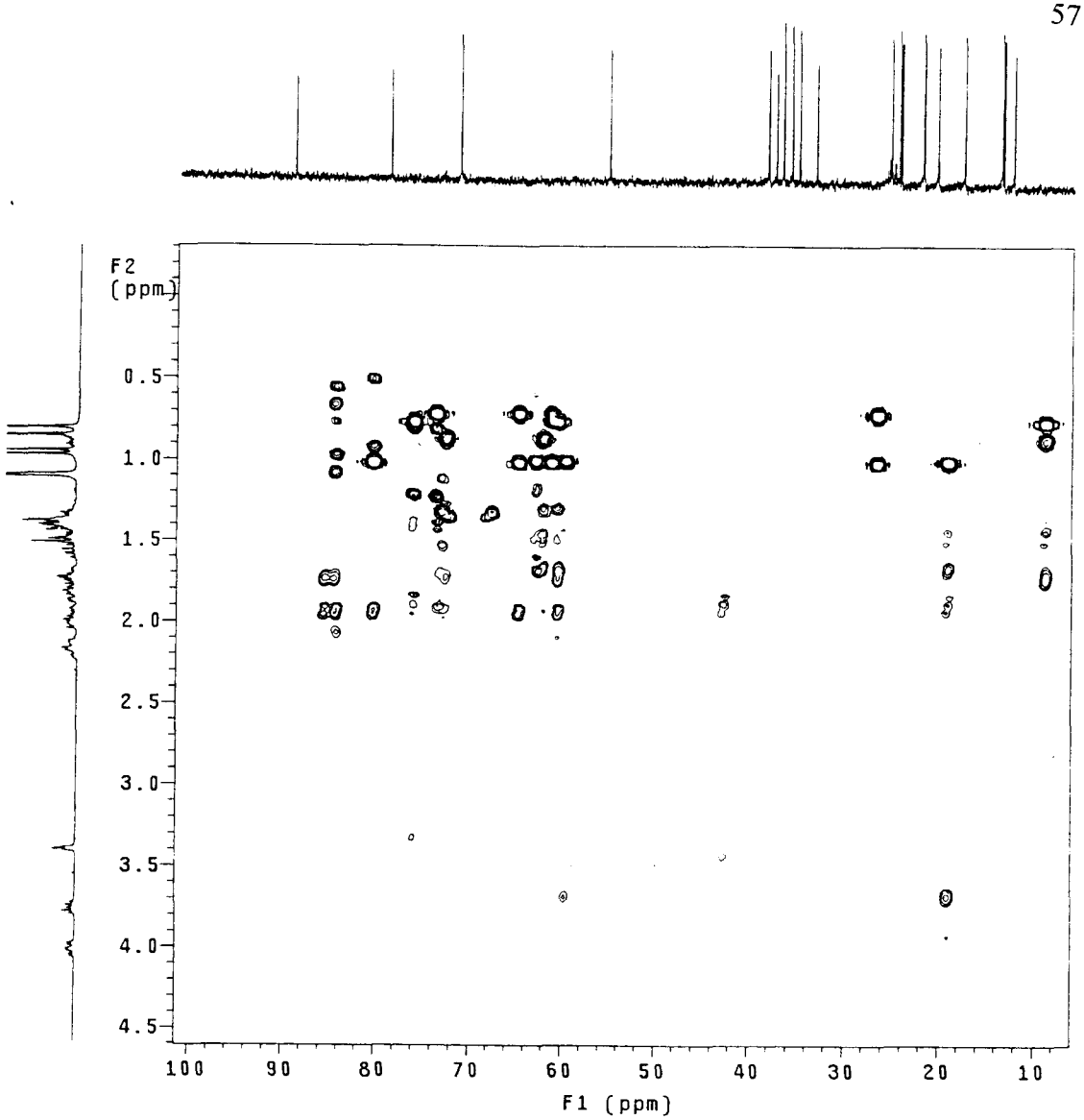


Figure B.6. HMBC spectrum of Compound I, in d_6-benzene.

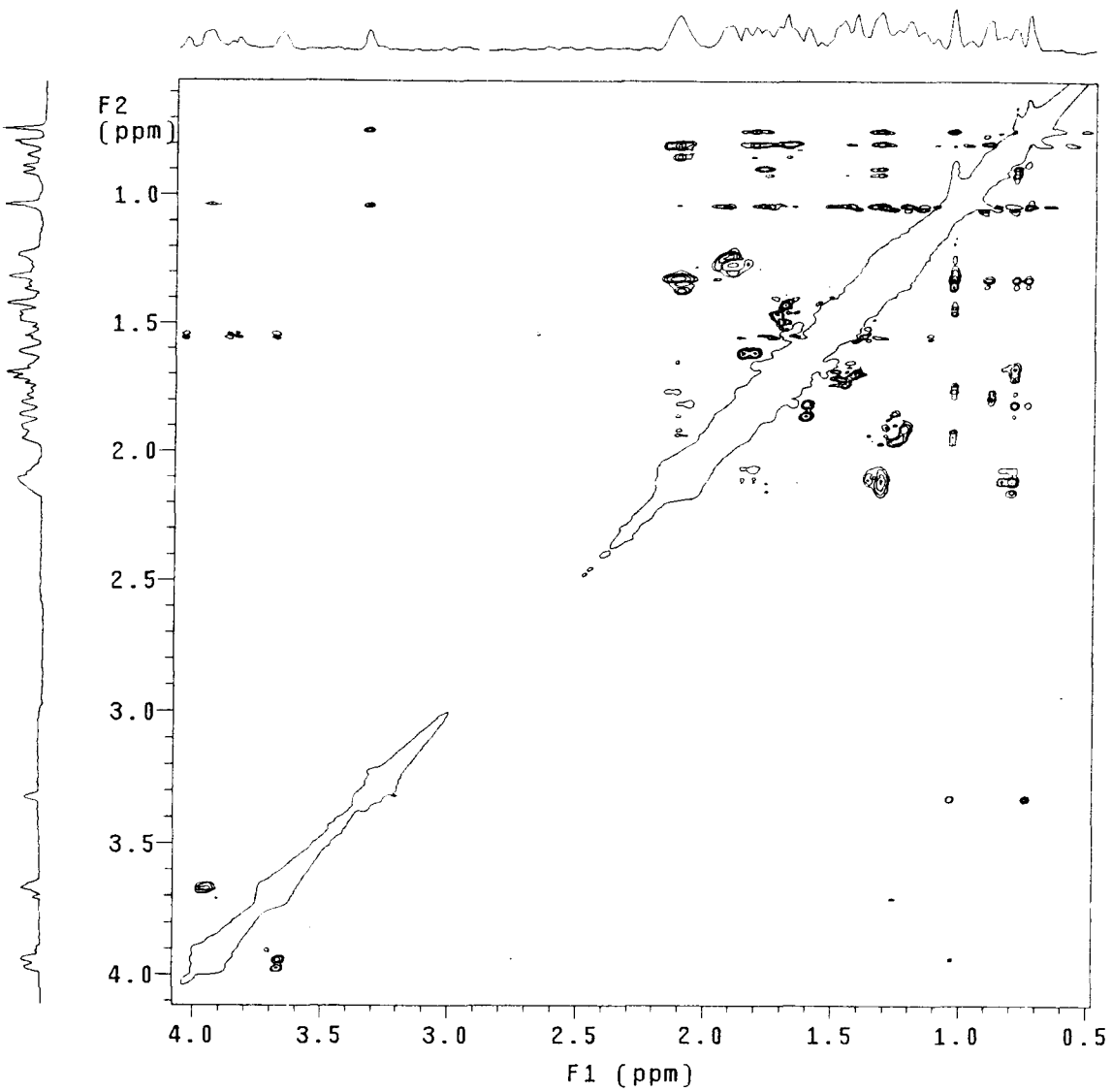


Figure B.7. NOESY spectrum of Compound I, in d_6-benzene.

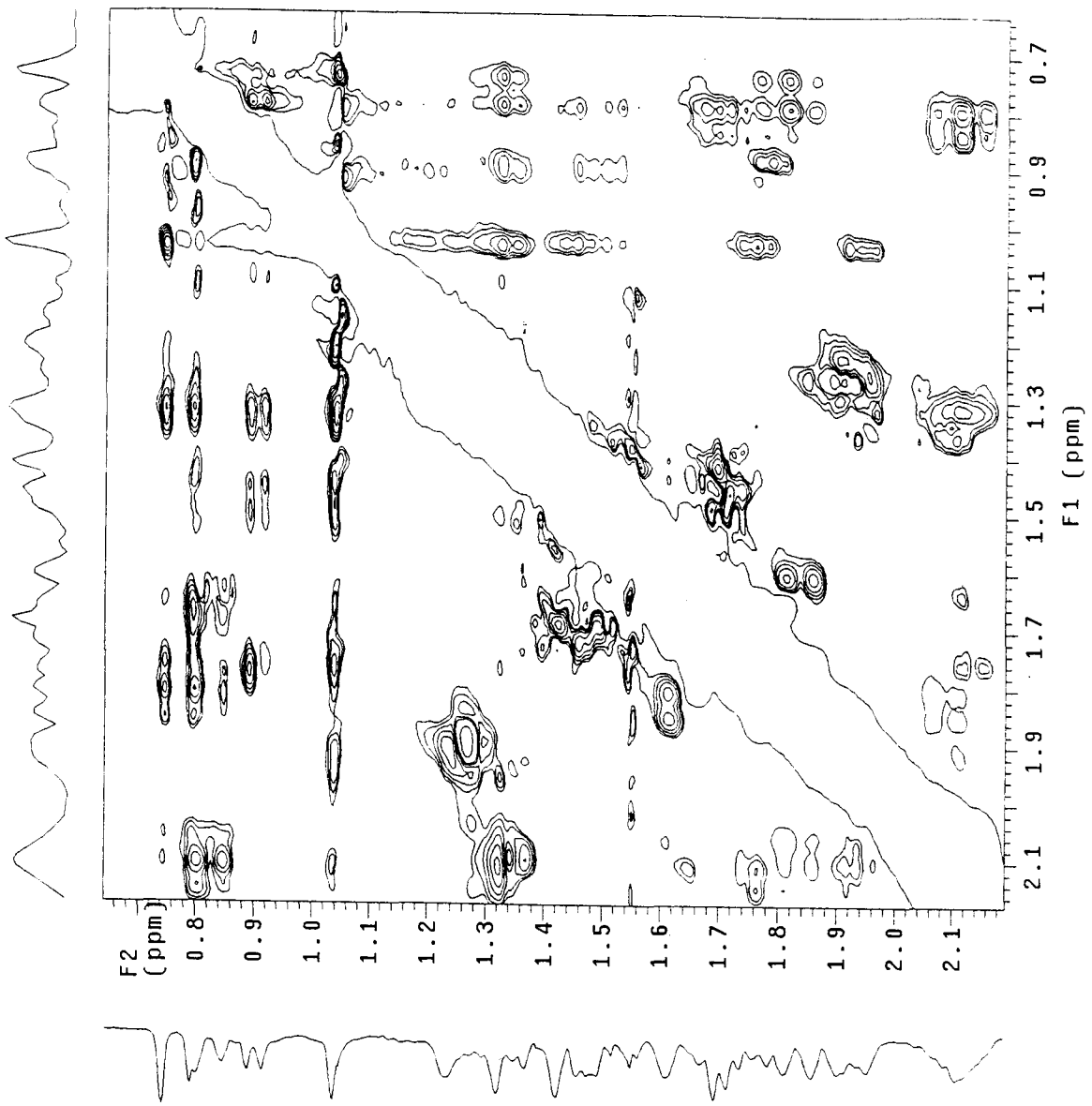


Figure B.8. NOESY spectrum of Compound I, in d_6-benzene, expanded.

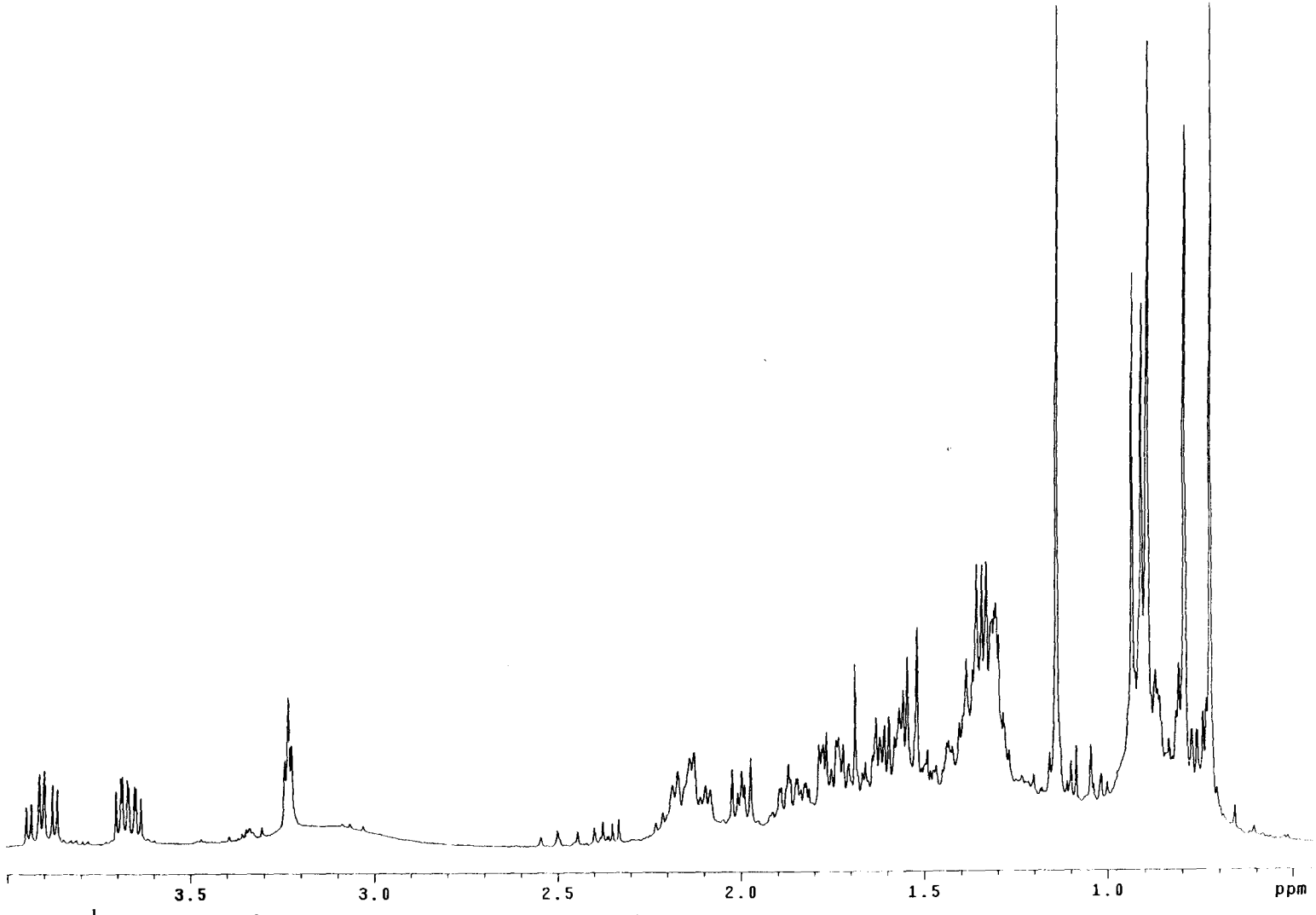
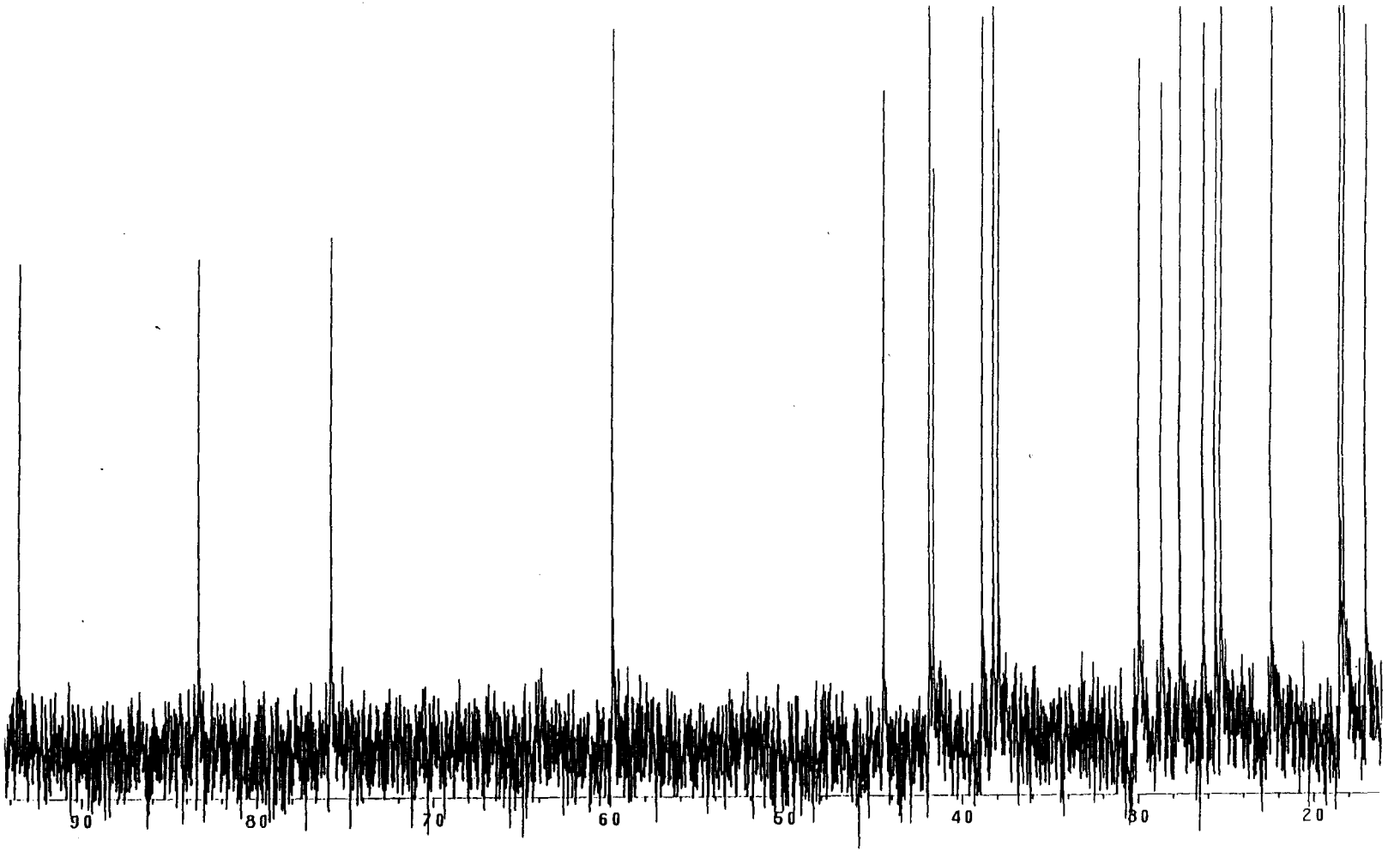


Figure B.9. ^{1}H spectrum of Compound II, in d_6-benzene.



B.10. ^{13}C NMR spectrum of Compound II, in d_6-benzene.

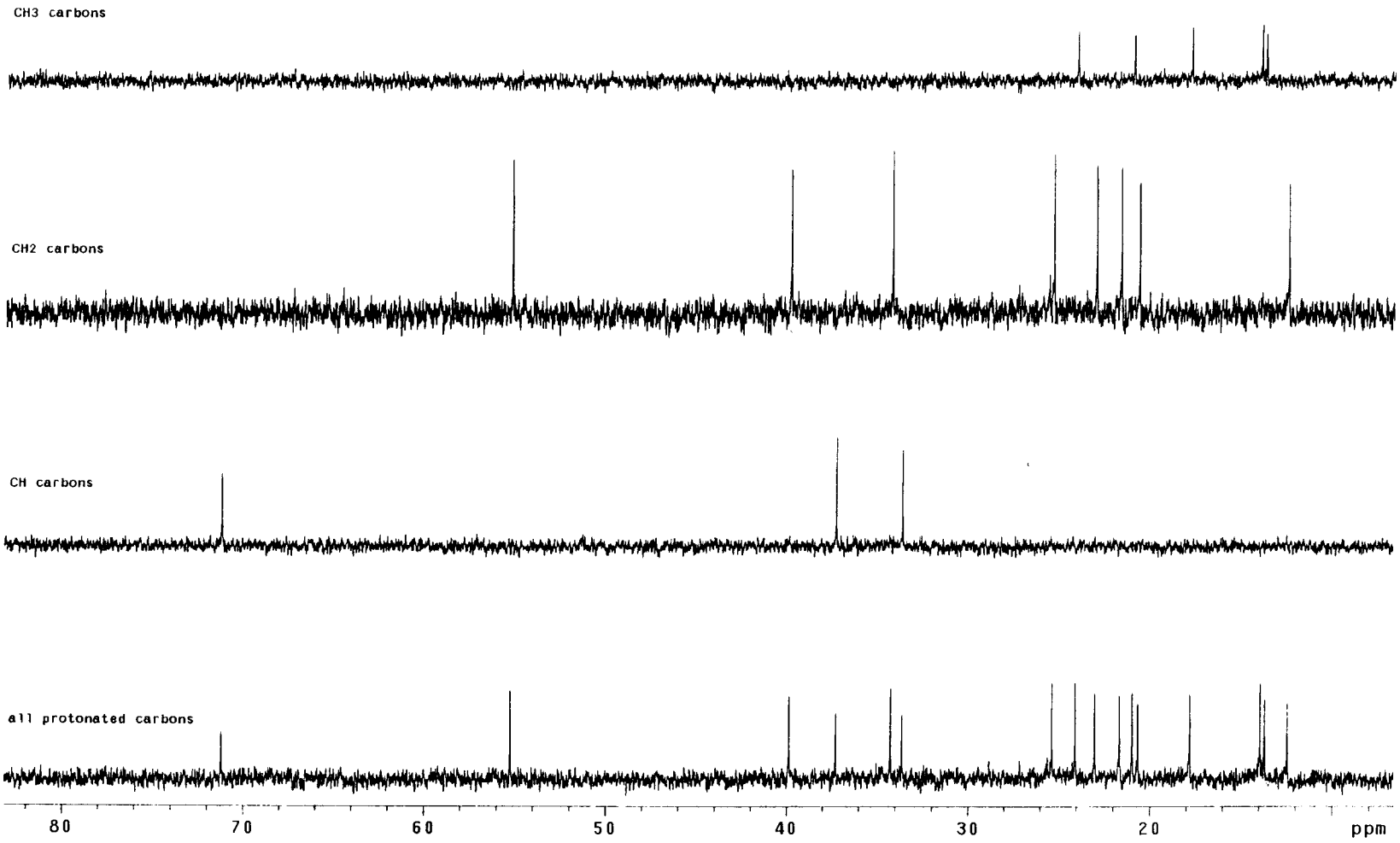


Figure B.11. DEPT spectrum of Compound II, in d_6-benzene.

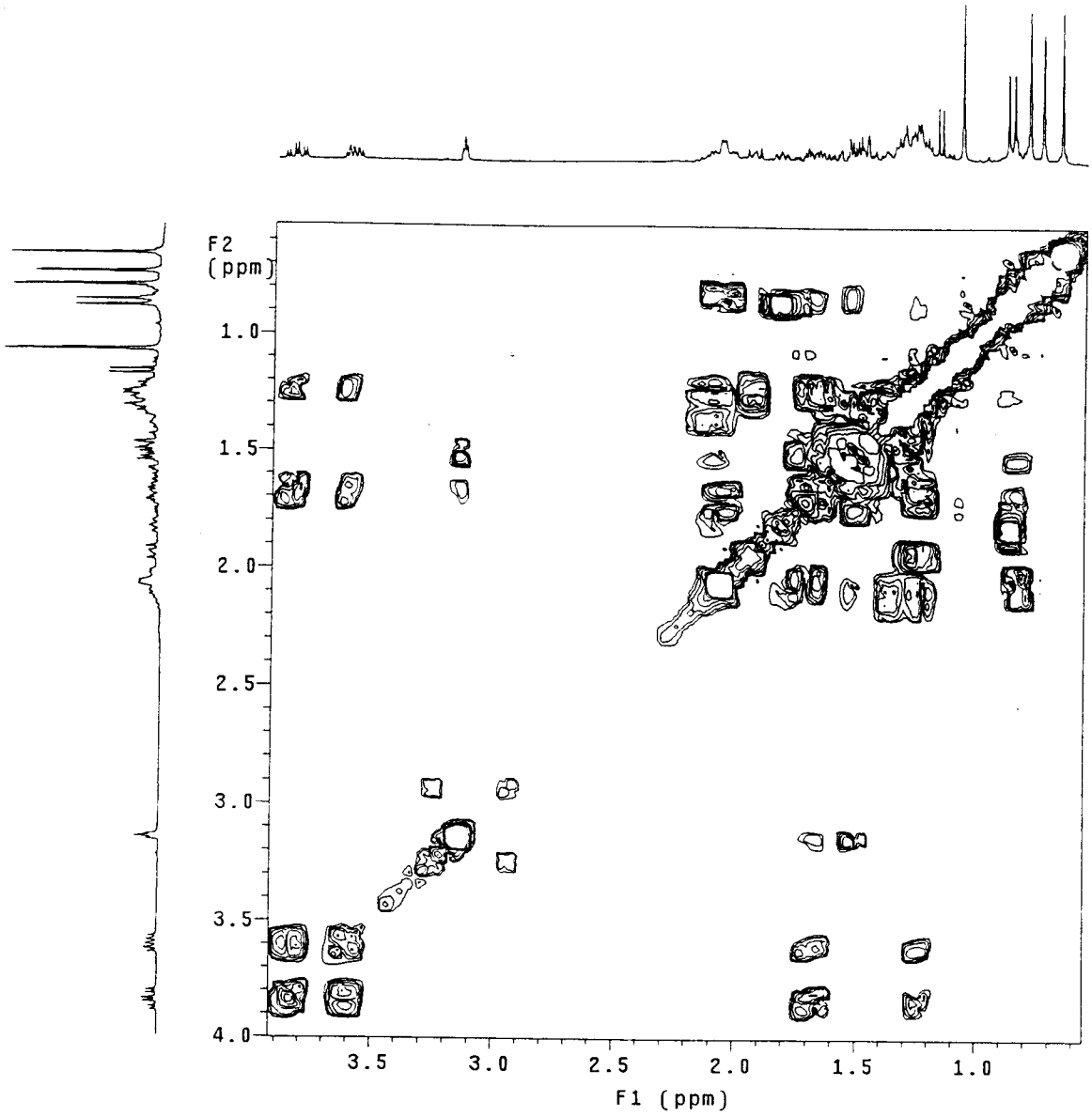


Figure B.12. gCOSY spectrum of Compound II, in d_6-benzene.

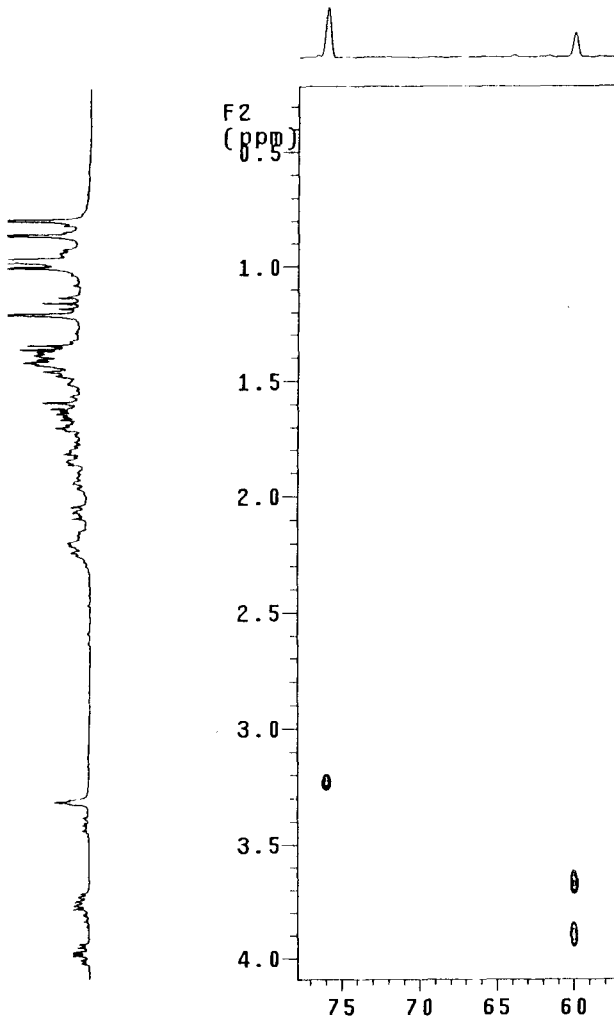
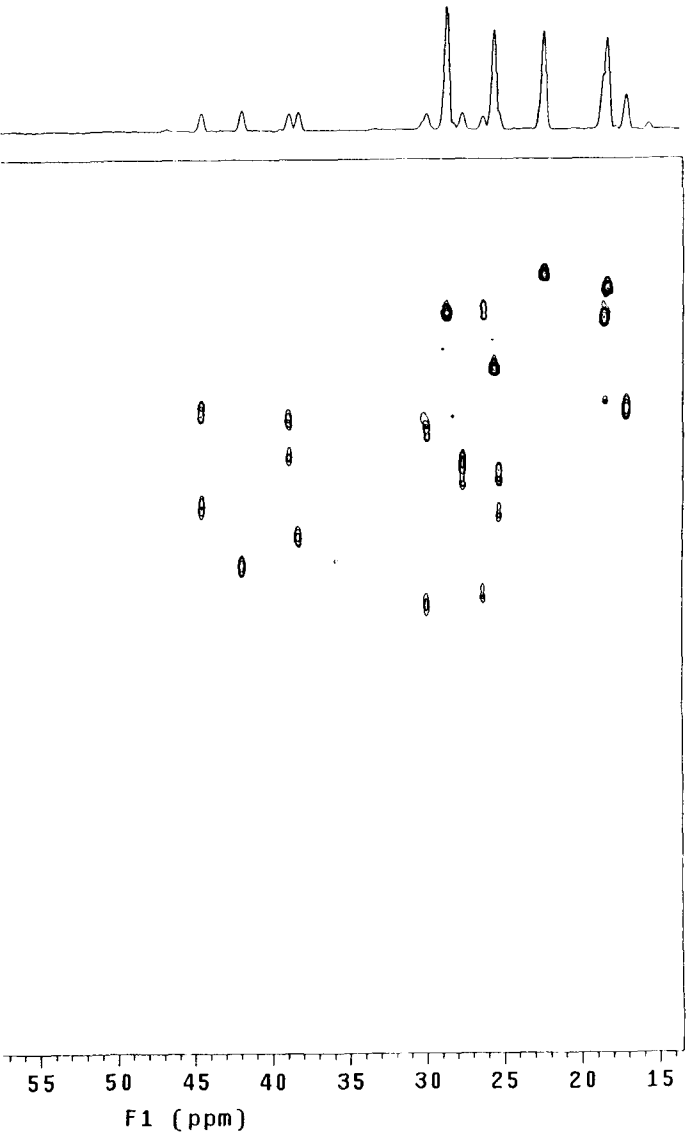


Figure B.13. HSQC spectrum of Compound II, in d_6-benzene.


55
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F1 (ppm)

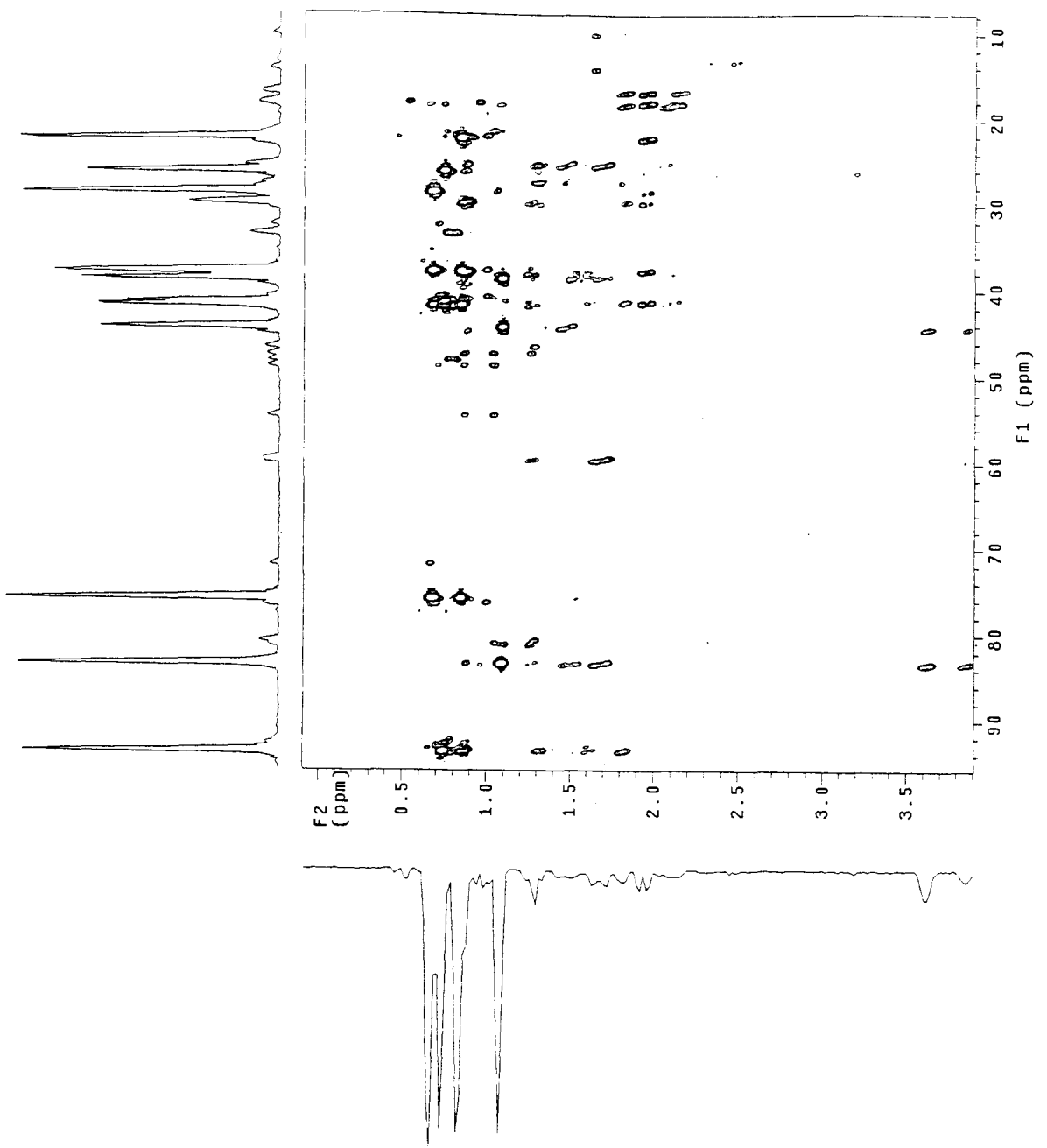


Figure B.14. HMBC spectrum of Compound II, in d_6-benzene.

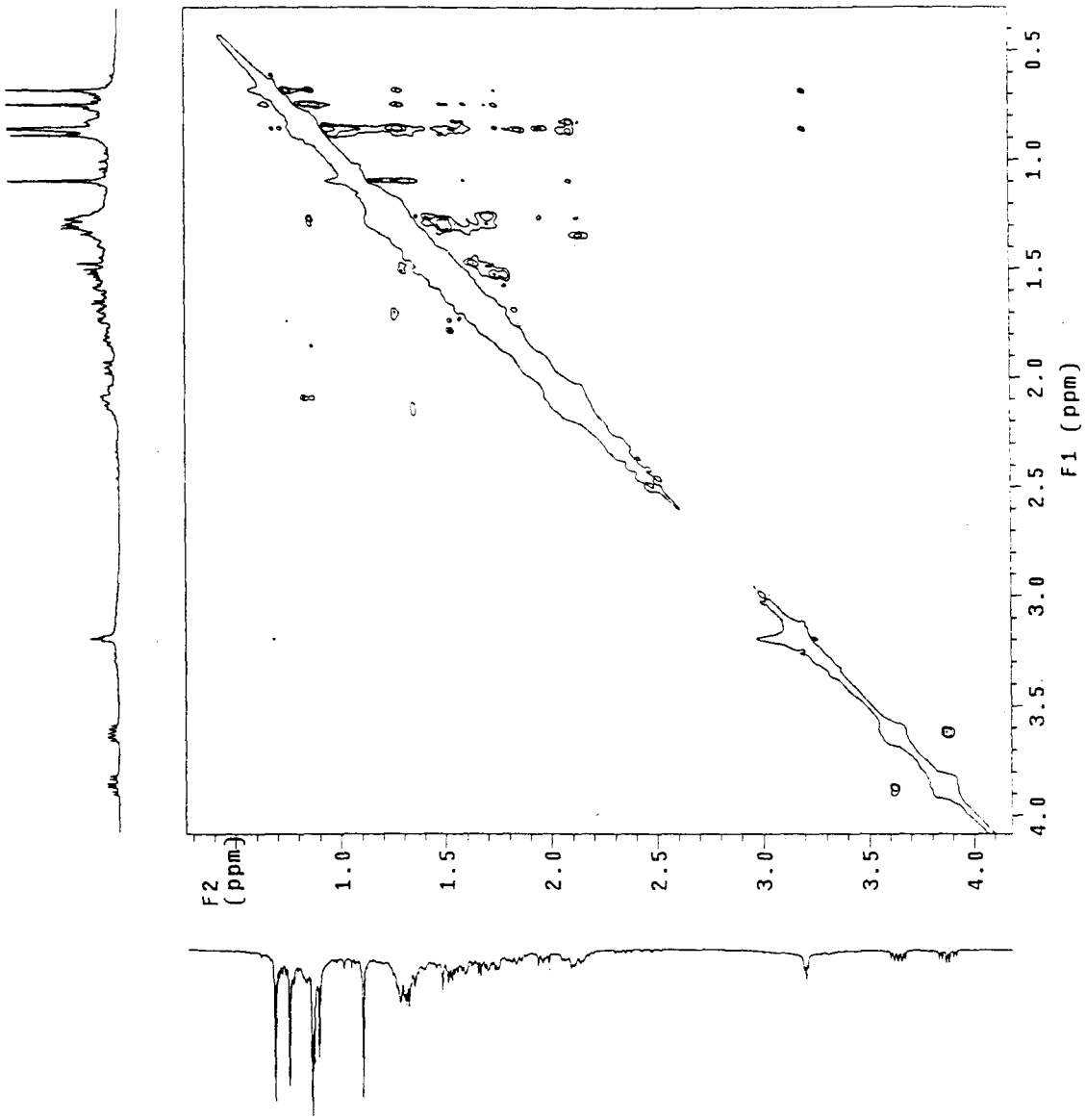


Figure B.15. NOESY spectrum of Compound II, in d_6-benzene.

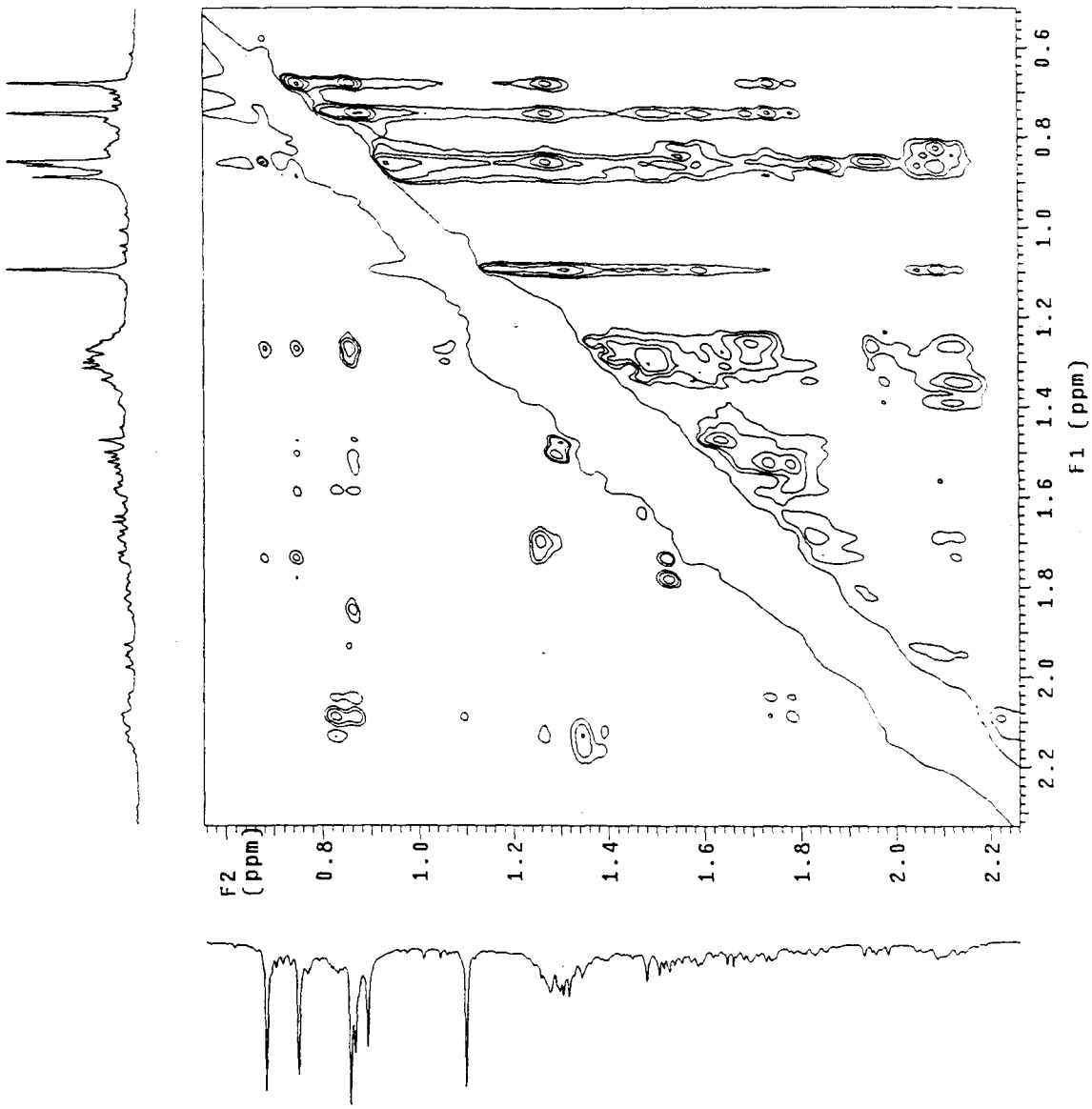


Figure B.16. NOESY spectrum of Compound II, in d_6-benzene, expanded.

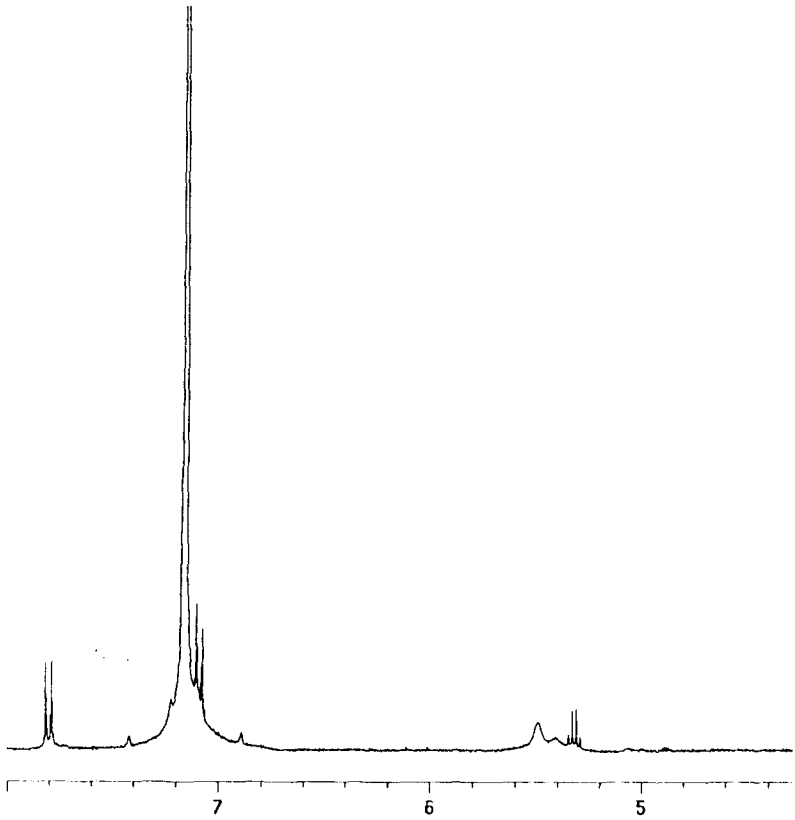
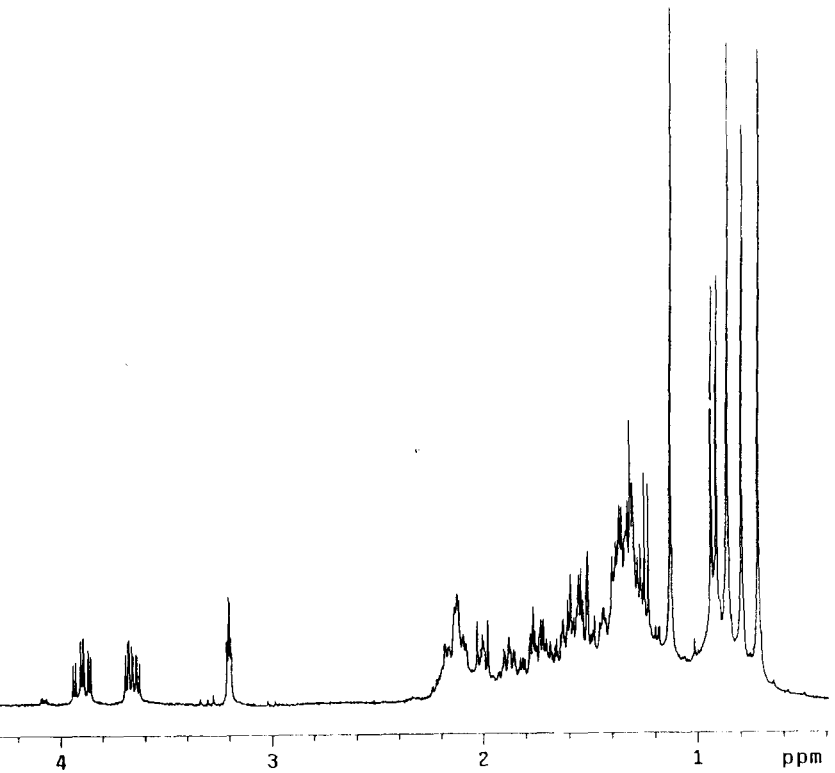


Figure B.17. ^{1}H spectrum of Compound IIa, in d_6-benzene.


4
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ppm

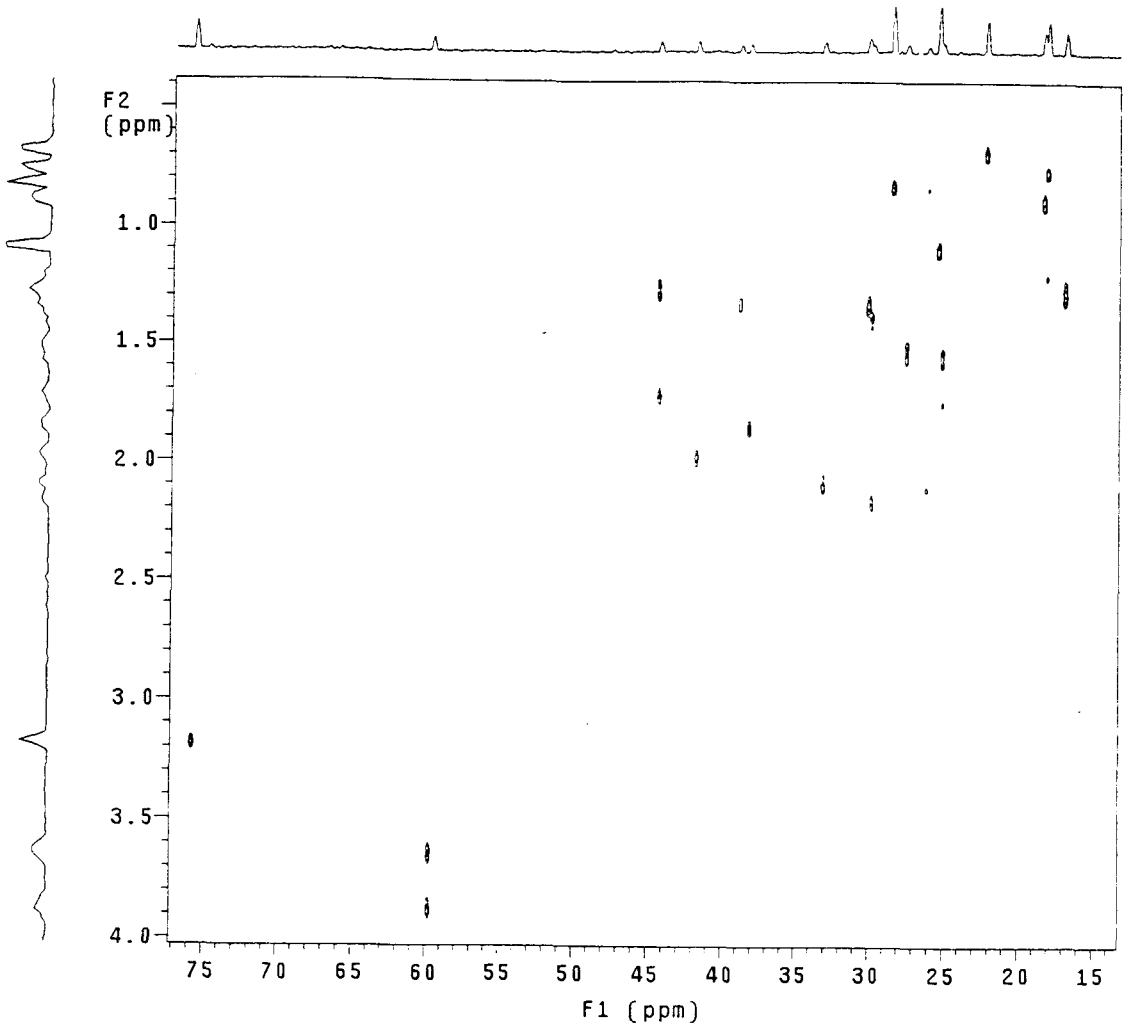


Figure B.18. HSQC spectrum of Compound IIa, in d_6-benzene.

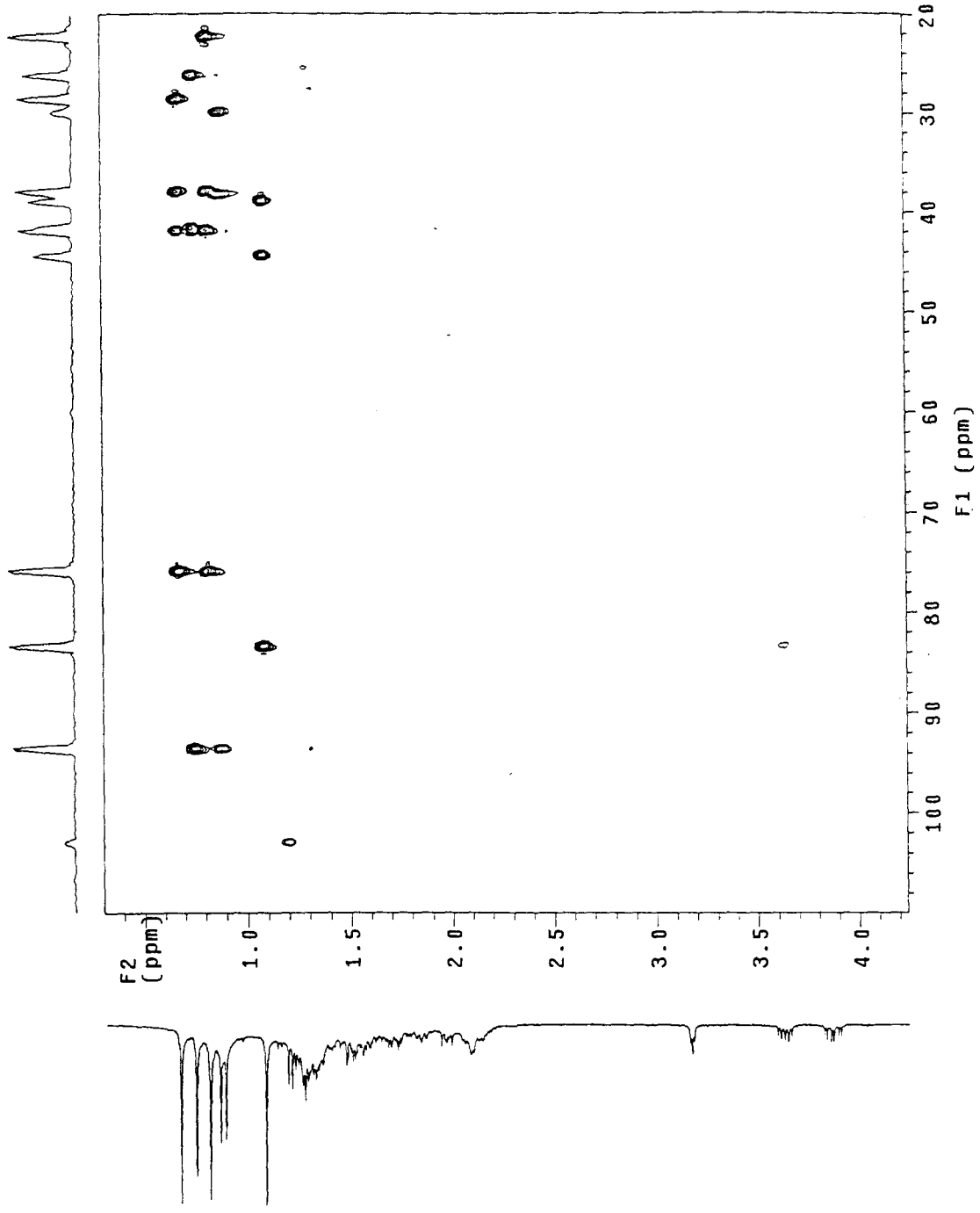
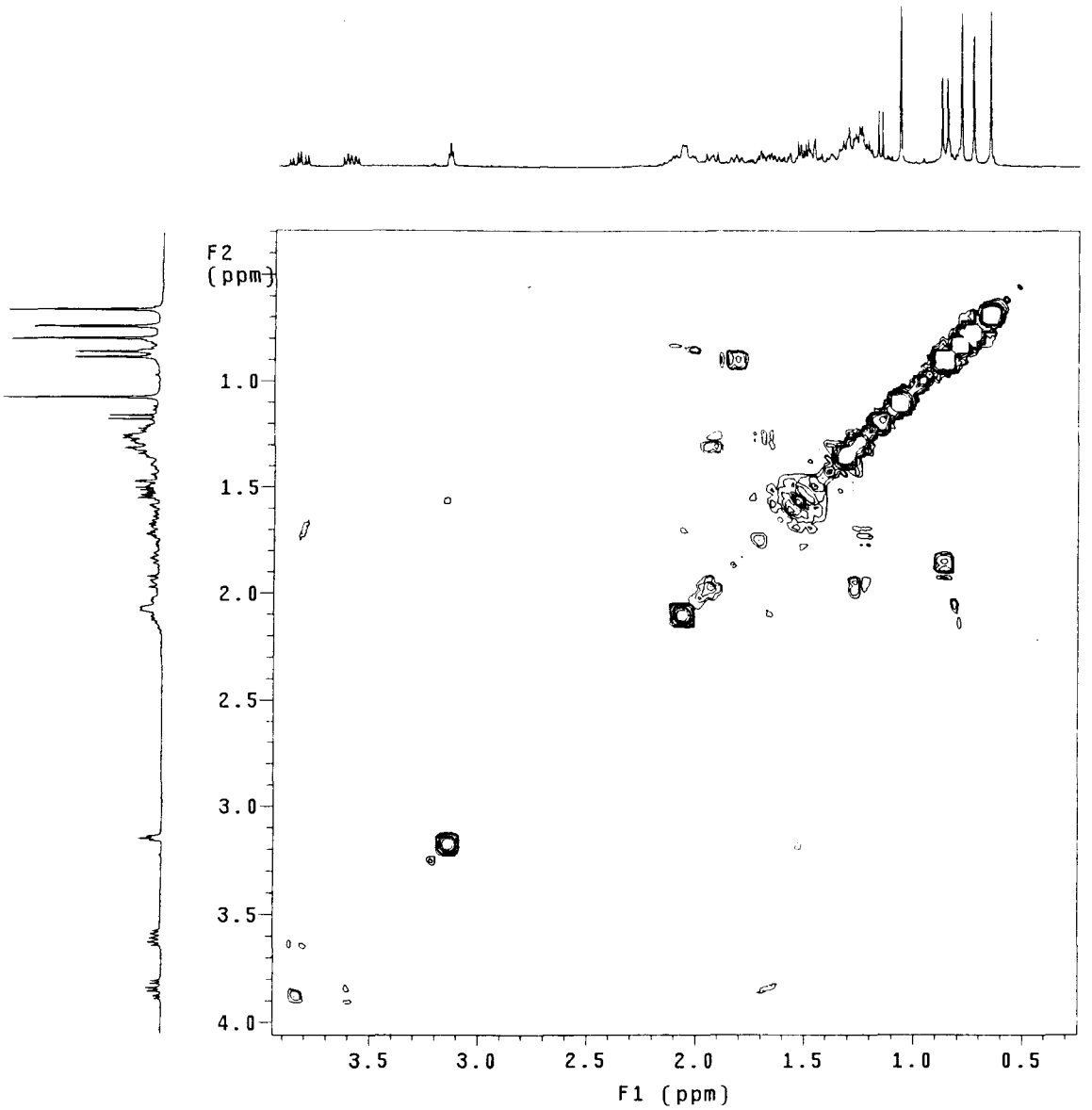


Figure B.19. HMBC spectrum of Compound IIa, in d_6-benzene.



B.20. gCOSY spectrum of Compound IIa, in d_6-benzene.

APPENDIX C. DATA FOR GROWTH STUDY

Table C.1. Dry weights of root and shoot samples, in milligrams.
*Failed Q-test; values omitted from average and standard deviation calculations.

Root	Age (wk)	Rinsed?	Wt. (mg)	Shoot	Age (wk)	Rinsed?	Wt. (mg)
100R	7	Yes	52.6	100S	7	Yes	390.3
101R	7	Yes	40.9	101S	7	Yes	321.8
102R	7	Yes	64.4	102S	7	Yes	315.2
103R	7	Yes	56.6	103S	7	Yes	333.3
104R	7	Yes	40.4	104S	7	Yes	276.9
105R	7	Yes	64.5	105S	7	Yes	320.3
106R	7	No	38.5	106S	7	No	264.4
107R*	7	No	*69.3	107S	7	No	395.4
108R	7	No	36.3	108S	7	No	322.1
109R	7	No	37.3	109S	7	No	272
110R	7	No	33.8	110S	7	No	280.3
111R	7	No	41.1	111S	7	No	358.3
160R	9	Yes	71.0	160S	9	Yes	356.8
161R	9	Yes	55.3	161S	9	Yes	406.1
162R	9	Yes	66.2	162S	9	Yes	309.5
163R	9	Yes	50.6	163S	9	Yes	359
164R	9	Yes	55.4	164S	9	Yes	346.8
165R	9	Yes	51.0	165S	9	Yes	340.3
166R	9	No	40.4	166S	9	No	384.9
167R	9	No	37.7	167S	9	No	378.5
168R	9	No	32.1	168S	9	No	270.5
169R	9	No	42.8	169S	9	No	312.5
170R	9	No	39.7	170S	9	No	279.4
171R	9	No	44.5	171S	9	No	280.5
136R	10	Yes	144.6	136S	10	Yes	567.8
137R	10	Yes	155.7	137S	10	Yes	566.9
138R	10	Yes	177.0	138S	10	Yes	511
139R	10	Yes	214.9	139S	10	Yes	708.5
140R	10	Yes	92.6	140S	10	Yes	634.7
141R	10	Yes	161.6	141S	10	Yes	598.6
142R	10	No	108.9	142S	10	No	582.6
143R*	10	No	*137.1	143S	10	No	538.9
144R	10	No	119.9	144S	10	No	549.7
145R	10	No	90.7	145S	10	No	508
146R	10	No	85.5	146S	10	No	393.5

Root	Age (wk)	Rinsed?	Wt. (mg)	Shoot	Age (wk)	Rinsed?	Wt. (mg)
147R	10	No	86.5	147S	10	No	355.4
148R	13	Yes	142.8	148S	13	Yes	483.4
149R	13	Yes	214.7	149S	13	Yes	688.6
150R	13	Yes	177.9	150S	13	Yes	402.2
151R	13	Yes	242.1	151S	13	Yes	491.3
152R	13	Yes	142.4	152S	13	Yes	400.6
153R	13	Yes	142.9	153S	13	Yes	707.9
154R	13	No	62.9	154S	13	No	205.6
155R	13	No	104.2	155S	13	No	112.5
156R	13	No	96.8	156S	13	No	477
157R	13	No	169.1	157S	13	No	493.3
158R	13	No	92.1	158S	13	No	342.5
159R	13	No	61.2	159S	13	No	410.4
112R	15	Yes	109.8	112S	15	Yes	456.2
113R	15	Yes	242.3	113S	15	Yes	872.3
114R	15	Yes	432.7	114S	15	Yes	794.4
115R	15	Yes	110.0	115S	15	Yes	731.9
116R	15	Yes	178.9	116S	15	Yes	726.9
117R	15	Yes	112.8	117S	15	Yes	581.9
118R	15	No	367.2	118S	15	No	639.7
119R	15	No	55.6	119S	15	No	283.2
120R	15	No	137.8	120S	15	No	443.8
121R	15	No	163.3	121S	15	No	321
122R	15	No	118.1	122S	15	No	453.6
123R	15	No	98.0	123S	15	No	413.3
124R	17	Yes	330.5	124S	17	Yes	759.7
125R	17	Yes	297.7	125S	17	Yes	1243.5
126R	17	Yes	586.1	126S	17	Yes	998.3
127R	17	Yes	287.4	127S	17	Yes	873.5
128R	17	Yes	325.8	128S	17	Yes	567.6
129R	17	Yes	197.3	129S	17	Yes	748.2
130R	17	No	107.3	130S	17	No	377.6
131R	17	No	281.0	131S	17	No	618.2
132R	17	No	191.6	132S	17	No	826.6
133R	17	No	98.4	133S	17	No	419.1
134R	17	No	285.8	134S	17	No	434.8
135R	17	No	278.1	135S	17	No	496
172R	19	Yes	310.5	172S	19	Yes	902
173R	19	Yes	325.5	173S	19	Yes	835.6

Root	Age (wk)	Rinsed?	Wt. (mg)	Shoot	Age (wk)	Rinsed?	Wt. (mg)
174R	19	Yes	334.1	174S	19	Yes	722.4
175R	19	Yes	523.6	175S	19	Yes	943.4
176R	19	Yes	392.6	176S	19	Yes	870.6
177R	19	Yes	380.8	177S	19	Yes	811.8
178R	19	No	717.5	178S	19	No	775
179R	19	No	450.3	179S	19	No	985.6
180R	19	No	361.3	180S	19	No	726.1
181R	19	No	486.9	181S	19	No	802.3
182R	19	No	195.1	182S	19	No	703.7
183R	19	No	151.6	183S	19	No	681.4

Table C.2. Concentrations of terpenes found in root and shoot samples, measured in ppm (per gram of dried plant material).

			Sesquiterpenes		Diterpenes	
Root	Age (wk)	Rinsed?	a-copaene	t-caryo-phyllene	Cmd IIIa & IIIb	Cmd I
100R	7	Yes	131.90	9.16	186.35	0.00
101R	7	Yes	3.05	0.00	33.79	0.00
102R	7	Yes	30.94	0.00	62.46	0.00
103R	7	Yes	41.27	0.00	163.41	0.00
104R	7	Yes	149.43	4.12	224.00	0.00
105R	7	Yes	9.76	0.00	0.00	0.00
106R	7	No	9.07	0.00	74.81	0.00
107R	7	No	19.20	0.91	61.72	0.00
108R	7	No	6.19	0.00	14.89	0.00
109R	7	No	0.00	0.00	8.91	0.00
110R	7	No	4.01	0.00	10.43	0.00
111R	7	No	62.85	0.96	41.55	0.00
160R	9	Yes	77.88	63.44	204.70	0.00
161R	9	Yes	38.07	4.70	41.01	0.00
162R	9	Yes	49.45	0.22	165.16	0.00
163R	9	Yes	5.04	0.00	31.07	0.00
164R	9	Yes	No data	No data	No data	No data
165R	9	Yes	45.05	1.00	69.90	0.00
166R	9	No	52.31	3.36	105.07	0.00
167R	9	No	9.54	0.00	6.17	0.00
168R	9	No	No data	No data	No data	No data
169R	9	No	86.42	7.10	72.85	0.00
170R	9	No	40.32	0.00	54.95	0.00
171R	9	No	No data	No data	No data	No data
136R	10	Yes	131.92	16.83	349.32	5.72
137R	10	Yes	30.69	0.00	286.65	0.00
138R	10	Yes	50.46	0.22	219.97	5.28
139R	10	Yes	40.92	0.00	97.04	0.00
140R	10	Yes	20.41	0.00	110.04	0.00
141R	10	Yes	90.76	5.25	397.55	0.00
142R	10	No	24.00	0.00	19.24	0.00
143R	10	No	55.75	10.08	16.15	0.00
144R	10	No	68.17	10.22	16.91	0.00
145R	10	No	55.66	16.52	0.00	0.00
146R	10	No	105.35	12.25	3.27	0.00
147R	10	No	23.33	13.06	3.24	0.00
148R	13	Yes	65.33	23.54	137.45	0.00

Root	Age (wk)	Rinsed?	Sesquiterpenes		Diterpenes	
			a-copaene	t-caryo-phyllene	Cmd IIIa & IIIb	Cmd I
149R	13	Yes	45.11	7.17	24.11	3.72
150R	13	Yes	145.44	8.01	200.57	4.61
151R	13	Yes	57.33	1.98	102.93	4.24
152R	13	Yes	34.53	0.00	160.19	0.00
153R	13	Yes	49.67	0.00	56.61	6.16
154R	13	No	116.74	0.00	134.43	0.00
155R	13	No	61.11	0.00	57.80	0.00
156R	13	No	67.71	0.00	119.54	0.00
157R	13	No	26.59	0.00	70.09	0.00
158R	13	No	59.66	0.00	108.89	0.00
159R	13	No	84.07	0.00	110.81	0.00
112R	15	Yes	No data	No data	No data	No data
113R	15	Yes	45.41	0.32	176.02	7.28
114R	15	Yes	100.44	5.51	375.45	4.03
115R	15	Yes	105.26	0.00	368.48	7.59
116R	15	Yes	496.99	21.36	1667.64	5.42
117R	15	Yes	107.80	1.71	746.13	9.87
118R	15	No	58.07	3.29	670.91	0.00
119R	15	No	18.34	0.00	216.28	0.00
120R	15	No	64.23	0.00	510.73	0.00
121R	15	No	281.10	24.78	2428.75	0.00
122R	15	No	44.20	0.00	342.84	0.00
123R	15	No	66.81	0.00	195.26	0.00
124R	17	Yes	162.82	14.27	294.47	5.54
125R	17	Yes	92.28	6.51	293.84	4.13
126R	17	Yes	167.30	17.96	402.66	1.87
127R	17	Yes	80.14	6.96	253.67	1.39
128R	17	Yes	50.68	3.14	386.77	3.45
129R	17	Yes	77.14	3.19	159.78	5.42
130R	17	No	50.95	0.00	117.46	8.88
131R	17	No	80.73	4.42	307.65	0.00
132R	17	No	78.69	2.62	123.31	8.82
133R	17	No	105.30	0.24	446.55	20.91
134R	17	No	44.05	1.25	158.08	0.00
135R	17	No	110.01	8.23	400.32	0.00
172R	19	Yes	102.23	7.10	121.69	4.80
173R	19	Yes	79.63	5.96	157.72	6.35
174R	19	Yes	43.83	2.10	146.13	3.81
175R	19	Yes	94.02	7.97	296.20	0.00

Root	Age (wk)	Rinsed?	Sesquiterpenes		Diterpenes	
			a-copaene	t-caryo-phyllene	Cmd IIIa & IIIb	Cmd I
176R	19	Yes	62.51	3.45	179.29	0.00
177R	19	Yes	6.34	12.59	264.53	0.00
178R	19	No	64.41	4.44	115.63	1.47
179R	19	No	42.15	1.91	142.47	2.17
180R	19	No	77.59	5.53	279.50	0.00
181R	19	No	55.50	3.64	100.43	4.19
182R	19	No	39.59	0.00	338.01	4.66
183R	19	No	37.97	0.00	142.20	0.00

Shoot	Age (wk)	Rinsed?	Sesquiterpenes		Diterpenes	
			a-copaene	t-caryo-phyllene	Cmd IIIa & IIIb	Cmd I
100S	7	Yes	1.81	1.68	0.00	0.00
101S	7	Yes	0.36	4.78	0.00	0.00
102S	7	Yes	4.35	6.27	0.00	0.00
103S	7	Yes	5.07	5.05	0.00	0.00
104S	7	Yes	3.24	6.31	0.00	0.00
105S	7	Yes	0.76	1.10	0.00	0.00
106S	7	No	No data	No data	No data	No data
107S	7	No	2.11	8.00	0.00	0.00
108S	7	No	1.33	3.34	0.00	0.00
109S	7	No	1.34	6.20	0.00	0.00
110S	7	No	0.00	1.76	0.00	0.00
111S	7	No	1.70	0.60	0.00	0.00
160S	9	Yes	9.83	3.87	1.48	0.00
161S	9	Yes	2.87	2.81	0.66	0.00
162S	9	Yes	3.39	3.98	1.95	0.00
163S	9	Yes	1.04	1.08	1.17	0.00
164S	9	Yes	27.48	4.07	3.44	0.00
165S	9	Yes	5.16	3.35	0.58	0.00
166S	9	No	0.62	1.56	0.26	0.00
167S	9	No	0.62	0.00	0.00	0.00
168S	9	No	3.58	0.52	0.00	0.00
169S	9	No	6.16	4.09	3.98	0.00
170S	9	No	8.52	3.69	0.63	0.00
171S	9	No	2.51	1.93	0.00	0.00
136S	10	Yes	6.18	2.29	0.00	0.00
137S	10	Yes	5.11	2.75	4.86	0.00
138S	10	Yes	5.32	2.38	0.00	0.00

Root	Age (wk)	Rinsed?	Sesquiterpenes		Diterpenes	
			a-copaene	t-caryo-phyllene	Cmd IIIa & IIIb	Cmd I
139S	10	Yes	10.14	4.21	2.41	0.00
140S	10	Yes	5.08	17.10	3.55	0.00
141S	10	Yes	10.39	5.82	3.96	0.00
142S	10	No	4.49	1.64	3.60	0.00
143S	10	No	14.18	4.58	4.11	0.00
144S	10	No	14.87	4.25	3.69	0.00
145S	10	No	9.94	5.10	0.00	0.00
146S	10	No	9.43	2.21	0.00	0.00
147S	10	No	5.68	15.83	0.00	0.00
148S	13	Yes	19.30	15.31	4.58	1.66
149S	13	Yes	14.07	3.83	1.65	1.24
150S	13	Yes	9.21	6.08	3.93	2.23
151S	13	Yes	29.40	17.12	5.22	0.00
152S	13	Yes	10.10	39.73	4.10	0.00
153S	13	Yes	15.18	2.47	1.69	0.44
154S	13	No	8.88	2.38	1.31	0.00
155S	13	No	12.73	7.74	0.00	0.00
156S	13	No	8.24	5.69	1.93	0.00
157S	13	No	4.98	2.30	0.59	0.00
158S	13	No	20.97	10.57	3.51	0.00
159S	13	No	8.15	1.43	0.87	0.00
112S	15	Yes	6.45	11.73	3.90	2.05
113S	15	Yes	13.84	18.99	8.16	0.97
114S	15	Yes	10.03	43.00	14.47	1.76
115S	15	Yes	12.92	9.40	5.25	1.88
116S	15	Yes	14.13	14.63	2.83	1.56
117S	15	Yes	11.56	3.33	5.29	2.18
118S	15	No	9.10	26.86	16.85	0.00
119S	15	No	5.18	5.44	2.84	0.00
120S	15	No	7.02	17.89	7.35	0.00
121S	15	No	6.85	2.90	4.97	0.00
122S	15	No	11.68	17.15	2.51	0.00
123S	15	No	9.05	6.17	0.89	0.81
124S	17	Yes	8.03	1.40	4.23	1.76
125S	17	Yes	13.57	4.31	5.14	0.79
126S	17	Yes	29.12	9.50	11.54	1.92
127S	17	Yes	12.18	3.77	5.05	1.35
128S	17	Yes	6.33	8.93	10.57	2.09
129S	17	Yes	6.26	1.39	2.07	1.39

Root	Age (wk)	Rinsed?	Sesquiterpenes		Diterpenes	
			a-copaene	t-caryo-phyllene	Cmd IIIa & IIIb	Cmd I
130S	17	No	6.16	4.92	1.88	0.91
131S	17	No	8.35	2.82	6.68	2.04
132S	17	No	12.50	3.39	2.66	1.14
133S	17	No	11.88	1.89	0.00	2.10
134S	17	No	13.15	6.09	3.42	2.13
135S	17	No	11.81	5.56	2.88	2.10
172S	19	Yes	15.72	8.41	3.44	1.65
173S	19	Yes	25.07	15.68	6.13	1.80
174S	19	Yes	10.51	3.61	5.09	1.21
175S	19	Yes	23.99	7.27	10.35	1.54
176S	19	Yes	14.31	10.66	5.07	1.53
177S	19	Yes	15.44	9.74	13.38	2.14
178S	19	No	12.85	4.57	4.12	1.62
179S	19	No	9.48	10.86	9.19	1.00
180S	19	No	27.34	12.07	8.63	1.62
181S	19	No	1.83	6.47	12.66	1.45
182S	19	No	9.85	4.57	2.75	1.53
183S	19	No	2.37	11.94	5.49	2.14